PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/57, 9/54 — A1

(11) International Publication Number: WO 96/09396

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/US95/05520

(22) International Filing Date: 28 April 1995 (28.04.95)

(30) Priority Data:
08/309,069 20 September 1994 (20.09.94) US

(71) Applicant: UNIVERSITY OF MARYLAND [US/US]; 4321 Hartwick Road, College Park, MD 20740 (US).

(72) Inventors: BRYAN, Philip, N.; 1720 Dublin Drive, Silver Spring, MD 20902 (US). ALEXANDER, Patrick, A.; 10402 Amherst Avenue, Silver Spring, MD 20902 (US). STRAUSBERG, Susan, L.; 403 Feather Rock Drive, Rockville, MD 20850 (US).

(74) Agents: REA, Teresa, Stanek et al.; Burns, Doane, Swecker & Mathis, Washington and Prince Streets, P.O. Box 1404, Alexandria, VA 22313-1404 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: CALCIUM FREE SUBTILISIN MUTANTS

(57) Abstract

Novel calcium free subtilisin mutants are taught, in particular subtilisins which have been mutated to eliminate amino acids 75-83 and which retain enzymatic activity and stability. Recombinant methods for producing same and recombinant DNA encoding for such subtilisin mutants are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

CALCIUM FREE SUBTILISIN MUTANTS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. GM42560 awarded by National Institute of Health.

GENERAL OBJECTS OF THE INVENTION

A general object of the invention is to provide subtilisin mutants which have been mutated such that they do not bind calcium.

Another object of the invention is to provide DNA sequences which upon expression provide for subtilisin mutants which do not bind calcium.

Another object of the invention is to provide subtilisin mutants which comprise specific combinations of mutations which provide for enhanced thermal stability.

Another object of the invention is to provide a method for the synthesis of a subtilisin mutant which does not bind calcium by the expression of a subtilisin DNA which comprises one or more substitution; deletion or addition mutations in a suitable recombinant host cell.

A more specific object of the invention is to provide class I subtilase mutants, in particular BPN′ mutants which have been mutated such that they do not bind calcium.

Another specific object of the invention is to provide DNA sequences which upon expression result in class I subtilase mutants, and in particular BPN′ mutants which do not bind calcium.

Another specific object of the invention is to provide a method for making subtilisin I-S1 or I-S2 mutants, and in particular BPN′ mutants which do not bind calcium by expression of a class I subtilase mutant DNA sequence, and more specifically a BPN′ DNA coding sequence which comprises one or more substitution, addition or deletion mutations in a suitable recombinant host cell.

Yet another specific object of the invention is to provide mutant subtilisin I-S1 or I-S2, and more specifically BPN' mutants which do not bind calcium and which further comprise particular combinations of mutations which provide for enhanced thermal stability, or which restore cooperativity to the folding reaction.

The subtilisin mutants of the present invention are to be utilized in applications where subtilisins find current usage. Given that these mutants do not bind calcium they should be particularly well suited for use in industrial environments which comprise chelating agents, e.g. detergent compositions, which substantially reduces the activity of wild-type calcium binding subtilisins.

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to subtilisin proteins which have been modified to eliminate calcium binding. More particularly, the present invention relates to novel subtilisin I-S1 and I-S2 subtilisin mutants, specifically BPN' mutants wherein the calcium A-binding loop has been deleted, specifically wherein amino acids 75-83 have been deleted, and which may additionally comprise one or more other mutations, e.g., subtilisin modifications, which provide for enhanced thermal stability and/or mutations which restore cooperativity to the folding reaction.

(2) Description of the Related Art

Subtilisin is an unusual example of a monomeric protein with a substantial kinetic barrier to folding and unfolding. A well known example thereof, subtilisin BPN' is a 275 amino acid serine protease secreted by *Bacillus amyloliquefaciens*. This enzyme is of considerable industrial importance and has been the subject of numerous protein engineering studies (Siezen et al., Protein Engineering 4:719-737 (1991); Bryan, Pharmaceutical Biotechnology 3(B):147-181 (1992); Wells et al., Trends Biochem. Sci. 13:291-297 (1988)). The amino acid sequence for subtilisin BPN' is known in the art and may be found in

Vasantha et al., J. Bacteriol. 159:811-819 (1984). The amino acid sequence as found therein is hereby incorporated by reference [SEQUENCE ID NO:1]. Throughout the application, when Applicants refer to the amino acid sequence of subtilisin BPN' or its mutants, they are referring to the amino acid sequence as listed therein.

Subtilisin is a serine protease produced by Gram positive bacteria or by fungi. The amino acid sequences of numerous subtilisins are known. (Siezen et al., Protein Engineering 4:719-737 (1991)). These include five subtilisins from *Bacillus* strains, for example, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, and mesenticopeptidase. (Vasantha et al., "Gene for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open-reading frame between the regions coding for signal sequence and mature protein," J. Bacteriol. 159:811-819 (1984); Jacobs et al., "Cloning sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*," Nucleic Acids Res. 13:8913-8926 (1985); Nedkov et al., "Determination of the complete amino acid sequence of subtilisin DY and its comparison with the primary structures of the subtilisin BPN', Carlsberg and amylosacchariticus," Biol. Chem. Hoppe-Seyler 366:421-430 (1985); Kurihara et al., "Subtilisin amylosacchariticus," J. Biol. Chem. 247:5619-5631 (1972); and Svendsen et al., "Complete amino acid sequence of alkaline mesentericopeptidase," FEBS Lett. 196:228-232 (1986)).

The amino acid sequences of subtilisins from two fungal proteases are known: proteinase K from *Tritirachium albam* (Jany et al., "Proteinase K from Tritirachium albam Limber," Biol. Chem. Hoppe-Seyler 366:485-492 (1985)) and thermomycolase from the thermophilic fungus, *Malbranchea pulchella* (Gaucher et al., "Endopeptidases: Thermomycolin," Methods Enzymol. 45:415-433 (1976)).

These enzymes have been shown to be related to subtilisin BPN', not only through their primary sequences and enzymological properties, but also by comparison of x-ray crystallographic data. (McPhalen et al., "Crystal and

molecular structure of the inhibitor eglin from leeches in complex with subtilisin Carlsberg," FEBS Lett., 188:55-58 (1985) and Pahler et al., "Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin," EMBO J., 3:1311-1314 (1984)).

Subtilisin BPN' is an example of a particular subtilisin gene secreted by *Bacillus amyloliquefaciens*. This gene has been cloned, sequenced and expressed at high levels from its natural promoter sequences in *Bacillus subtilis*. The subtilisin BPN' structure has been highly refined (R = 0.14) to 1.3 Å resolution and has revealed structural details for two ion binding sites (Finzel et al., J. Cell. Biochem. Suppl. 10A:272 (1986); Pantoliano et al., Biochemistry 27:8311-8317 (1988); McPhalen et al., Biochemistry 27: 6582-6598 (1988)). One of these (site A) binds Ca^{2+} with high affinity and is located near the N-terminus, while the other (site B) binds calcium and other cations much more weakly and is located about 32 Å away (Figure 1). Structural evidence for two calcium binding sites was also reported by Bode et al., Eur. J. Biochem. 166:673-692 (1987) for the homologous enzyme, subtilisin Carlsberg.

Further in this regard, the primary calcium binding site in all of the subtilisins in groups I-S1 and I-S2 (Siezen et al., 1991, Table 7) are formed from almost identical nine residue loops in the identical position of helix C. X-ray structures of the I-S1 subtilisins BPN' and Carlsberg, as well as the I-S2 subtilisin Savinase, have been determined to high resolution. A comparison of these structures demonstrates that all three have almost identical calcium A-sites.

The x-ray structure of the class I subtilase, thermitase from *Thermoactinomyces vulgaris*, is also known. Though the overall homology of BPN' to thermitase is much lower than the homology of BPN' to I-S1 and I-S2 subtilisins, thermitase has been shown to have an analogous calcium A-site. In the case of thermitase, the loop is a ten residue interruption at the identical site in helix C.

Calcium binding sites are common features of extracellular microbial proteases probably because of their large contribution to both thermodynamic and

kinetic stability (Matthews et al., J. Biol. Chem. 249:8030-8044 (1974); Voordouw et al., Biochemistry 15:3716-3724 (1976); Betzel et al., Protein Engineering 3:161-172 (1990); Gros et al., J. Biol. Chem. 266:2953-2961 (1991)). The thermodynamic and kinetic stability of subtilisin is believed to be necessitated by the rigors of the extracellular environment into which subtilisin is secreted, which by virtue of its own presence is protease-filled. Accordingly, high activation barriers to unfolding may be essential to lock the native conformation and prevent transient unfolding and proteolysis.

Unfortunately, the major industrial uses of subtilisins are in environments containing high concentrations of metal chelators, which strip calcium from subtilisin and compromise its stability. It would, therefore, be of great practical significance to create a highly stable subtilisin which is independent of calcium.

The present inventors have previously used several strategies to increase the stability of subtilisin to thermal denaturation by assuming simple thermodynamic models to approximate the unfolding transition (Pantoliano et al., Biochemistry 26:2077-2082 (1987); Pantoliano et al., Biochemistry 27:8311-8317 (1988); Pantoliano et al., Biochemistry 28:7205-7213 (1989); Rollence et al., CRC Crit. Rev. Biotechnol. 8:217-224 (1988). However, improved subtilisin mutants which are stable in industrial environments, e.g., which comprise metal chelators, and which do not bind calcium, are currently not available.

OBJECTS AND SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to provide mutated or modified subtilisin enzymes, e.g., class I subtilases, which have been modified to eliminate calcium binding. As used in this invention, the term "mutated or modified subtilisin" is meant to include any serine protease enzyme which has been modified to eliminate calcium binding. This includes, in particular, subtilisin BPN' and serine proteases which are homologous to subtilisin BPN', in particular class I subtilases. However, as used herein, and under the definition of mutated or modified subtilisin enzyme, the mutations of this invention may be

introduced into any serine protease which has at least 50%, and preferably 80% amino acid sequence identity with the sequences referenced above for subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, mesenticopeptidase, thermitase, or Savinase and, therefore, may be considered homologous.

The mutated subtilisin enzymes of this invention are more stable in the presence of metal chelators and may also comprise enhanced thermal stability in comparison to native or wild-type subtilisin. Thermal stability is a good indicator of the overall robustness of a protein. Proteins of high thermal stability often are stable in the presence of chaotropic agents, detergents, and under other conditions, which normally tend to inactivate proteins. Thermally stable proteins are, therefore, expected to be useful for many industrial and therapeutic applications in which resistance to high temperature, harsh solvent conditions or extended shelf-life is required.

It has been further discovered that combining individual stabilizing mutations in subtilisin frequently results in approximately additive increases in the free energy of stabilization. Thermodynamic stability has also been shown to be related to resistance to irreversible inactivation at high temperature and high pH. The single-site changes of this invention individually do not exceed a 1.5 Kcal/mol contribution to the free energy of folding. However, these small incremental increases in the free energy of stabilization result in dramatic increases in overall stability when mutations are combined, since the total free energy of folding for most proteins is in the range of 5-15 Kcals/mol (Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman and Company, New York (1984)).

X-ray crystallographic analysis of several combination mutants reveals that conformational changes associated with each mutation tend to be highly localized with minimal distortion of the backbone structure. Thus, very large increases in stability can be achieved with no radical changes in the tertiary protein structure and only minor independent alterations in the amino acid

sequence. As previously suggested (Holmes et al, J. Mol. Biol. 160:623 (1982)), contributions to the free energy of stabilization can be gained in different ways, including improved hydrogen bonding and hydrophobic interactions in the folded form and decreased chain entropy of the unfolded enzyme. This is significant since thermostable enzymes generally have more extended half-lives at broader temperature ranges, thereby improving bio-reactor and shelf-life performance.

As noted *supra*, the invention provides subtilisin mutants which comprise one or more deletion, substitution or addition mutations which provide for the elimination of calcium binding. Preferably, this will be effected by deletion, substitution or insertion of amino acids into the calcium A-site, which in the case of class I subtilases comprises 9 amino acid residues in helix C. In the case of subtilisin BPN', the subtilisin mutants will preferably comprise one or more addition, deletion or substitution mutations of the amino acids at positions 75-83, and most preferably will comprise the deletion of amino acids 75-83, of SEQUENCE ID NO:1. The deletion of amino acids 75-83 has been discovered to effectively eliminate calcium binding to the resultant subtilisin mutant while still providing for subtilisin BPN' proteins having enzymatic activity.

Such subtilisin mutants lacking amino acids 75-83 of SEQUENCE ID NO:1 may further include one or more additional amino acid mutations in the sequence, e.g., mutations which provide for reduced proteolysis. It is another object of the invention to produce subtilisin mutants lacking calcium binding activity which have been further mutated to restore cooperativity to the folding reaction and thereby enhance proteolytic stability. It is another object of the invention to provide thermostable subtilisin mutants which further do not bind calcium and comprise specific combinations of mutations which provide for substantially enhanced thermal stability.

In particular, the subtilisin mutants of the present invention will include subtilisins from *Bacillus* strains, such as subtilisin BPN', subtilisin Carlsberg,

subtilisin DY, subtilisin amylosacchariticus and subtilisin mesenticopeptidase, which comprise one or more deletion, substitution or addition mutations.

The present invention further provides for subtilisin mutants lacking amino acids 75-83 of SEQUENCE ID NO:1, which have new protein-protein interactions engineered in the regions around the deletion leading to large improvements in stability. More specifically, mutations at ten specific sites in subtilisin BPN' and its homologues are provided, seven of which individually, and in combination, have been found to increase the stability of the subtilisin protein. Improved calcium-free subtilisins are thus provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. X-ray Crystal structure of S15 subtilisin

A. α-carbon plot shows the positions of mutations as noted. The numbering of wild type subtilisin is kept. Dotted spheres show the position of calcium at the weak ion binding site (B-site) and the former position of the high affinity binding site (A-site). The A-site loop (dashed line) is absent in this mutant. N- and C- termini are indicated. The N-terminus is disordered (dotted line).

B. Close-up view of the A-site deletion. The loop from S12 subtilisin is shown as a dotted line with the continuous helix of S15. Superimposed is the 3* sigma difference electron density (FO12-FO15, phases from S15) showing the deleted A-site loop.

Figure 2. X-ray crystal structure of the calcium A-site region of S12 subtilisin. Calcium is shown as a dotted sphere with one-half the van der Waals radius. Dashed lines are coordination bonds, while dotted lines represent hydrogen bonds under 3.2 Å.

Figure 3. Differential Scanning Calorimetry. The calorimetric scans of apo-S12 (T_m = 63.5°C) and S15 (T_m = 63.0°C) are shown. Measurements were performed with a Hart 7707 DSC (differential scanning calorimetry) heat conduction scanning microcalorimeter as described (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). Sample conditions were 50 mM of glycine, a pH of 9.63, a scan rate of 0.5°C/min. Excess heat capacity is measured in units of μJ/°. The calorimeter ampoules contained 1.78 mg of protein.

Figure 4. Titration calorimetry of subtilisin S11. The heat of calcium binding for successive additions of calcium are plotted vs. the ratio of [Ca]/[P]. The data are best fit by a calculated binding curve assuming a binding constant of 7×10^6 and ΔH equal to 11.3 kcal/mol using equation (1) from the text. For comparison, calculated curves assuming $K_a = 1 \times 10^6$ and 1×10^8 are also shown. In this titration, [P] = 100μM and the temperature was 25°C.

Figure 5. Kinetics of calcium dissociation from subtilisin S11 as a function of temperature. 1μM subtilisin S11 was added to 10μM Quin2 at time = 0. Calcium dissociates from subtilisin and binds to Quin2 until a new equilibrium is achieved. The rate of calcium dissociation is followed by the increase in fluorescence of Quin2 when it binds to calcium.

A. The log of the percent of the protein bound to calcium is plotted vs. time. The kinetics of dissociation at four temperatures are shown. The dissociation follows first order kinetics for the first 25% of the reaction. As this is well before equilibrium is approached, reassociation of calcium can be neglected.

B. Temperature dependence of the rate of calcium dissociation from S15 subtilisin in the presence of excess Quin2, pH 7.4 and over a temperature range of 25-45°C. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs. the reciprocal of the

absolute temperature. The line is fit according to equation (3) in the text with T_0 = 298 K.

Figure 6. Analysis of subtilisin refolding monitored by circular dichroism (CD).

A. CD spectra are shown for S15 as follows: (1) S15 in 25 mM H_3PO_4 at pH 1.85; (2) S15 denatured at pH 1.85 and then neutralized to pH 7.5 by the addition of NaOH; (3) S15 denatured at pH 1.85 and neutralized to pH 7.5, 30 minutes after the addition of KCl to 0.6 M; and (4) Native S15 subtilisin. Protein concentrations of all samples was 1 μM.

B. Kinetics of refolding of S15. Samples were denatured at pH 1.85 and then the pH was adjusted to 7.5. At time 0, KCl was added to the denatured protein. Recovery of native structure was followed at 222 nm at KCl concentrations of 0.3 M and 0.6 M. The 0.6 M sample after 30 minutes of refolding was then used to record the corresponding spectrum in part A.

Figure 7. Kinetics of refolding of S15 as a function of ionic strength.

A. The log of the percent unfolded protein is plotted vs. time. The kinetics of refolding are shown at four ionic strengths. The amount of refolding was determined by circular dichroism (CD) from the increase in negative ellipticity at 222 nm. 100% folding is determined from the signal at 222 nm for native S15 at the same concentration and 0% folding is determined from the signal for acid-denatured S15. The refolding approximately follows first order kinetics for the first 90% of the reaction. Refolding was carried out at 25°C.

B. The log of first order rate constants for refolding obtained by CD or fluorescence measurements at 25°C were plotted as a function of log of ionic strength. Ionic strength was varied from I=0.25 to I=1.5. The rate of refolding increases linearly with log I. A ten-fold increase in I results in an approximately 90-fold increase in the refolding rate.

Figure 8. Temperature dependence of the refolding rate of S15 subtilisin in 0.6 M KCl, 23 nM KPO_4 pH 7.3. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs. the reciprocal of the absolute temperature. The line is fit according to equation 3 in the text with T_0=298 K.

Figure 9. X-ray crystal structure of the weak ion binding region of S15 subtilisin. Coordination bonds are shown as dashed lines. Note the preponderance of charged amino acids.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed *supra*, calcium binding contributes substantially to the thermo-dynamic and kinetic-stability of extracellular microbial proteases. Moreover, with respect to subtilisin, high activation barriers to unfolding may be essential to retain the native conformation and to prevent transient unfolding and proteolysis given the protease-filled environment where subtilisin is secreted and as a result of auto-degradation. The unfolding reaction of subtilisin can be divided into two parts as follows:

$$N(Ca) \leftrightarrow N \leftrightarrow U$$

where N(Ca) is the native form of subtilisin with calcium bound to the high affinity calcium-binding site A (Finzel et al., J. Cell. Biochem. Suppl. 10A:272 (1986); Pantoliano et al., Biochemistry 27:8311-8317 (1988); McPhalen et al., Biochemistry 27:6582-6598 (1988)); N is the folded protein without calcium bound; and U is the unfolded protein. Subtilisin is a relatively stable protein whose stability is in large part mediated by the high affinity calcium site (Voordouw et al., Biochemistry 15:3716-3724 (1976); Pantoliano et al., Biochemistry 27:8311-8317 (1988)). The melting temperature of subtilisin at pH 8.0 in the presence of μmolar concentrations of calcium is approximately 75°C and approximately 56°C in the presence of excess EDTA (Takehashi et al., Biochemistry 20:6185-6190 (1981); Bryan et al., Proc. Natl. Acad. Sci. USA,

83:3743-3745 (1986b)). Previous calorimetric studies of the calcium-free (apoenzyme, i.e., protein portion of enzyme) form of subtilisin indicated that it is of marginal stability at 25°C with a ΔG of unfolding of <5 kcal/mol (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). Because calcium is such an integral part of the subtilisin structure, the apoenzyme is thought to be a folding intermediate of subtilisin.

In order to independently examine the two phases of the folding process, the present inventors constructed a series of mutant subtilisins. First, all proteolytic activity was eliminated in order to prevent auto-degradation from occurring during the unfolding and refolding reactions. This may be accomplished, for example, by converting the active-site serine 221 to cysteine.[1] This mutation has little effect on the thermal denaturation temperature of subtilisin, but reduces peptidase activity of subtilisin by a factor of approximately 3×10^4 (Abrahmsen et al., Biochemistry 30:4151-4159 (1991)). This mutant, therefore, allows the folding of subtilisin to be studied without the complications of proteolysis. In the present specification, a shorthand for denoting amino acid substitutions employs the single letter amino acid code of the amino acid to be substituted, followed by the number designating where in the amino acid sequence the substitution will be made, and followed by the single letter code of the amino acid to be inserted therein. For example, S221C denotes the substitution of serine 221 to cysteine. The subtilisin mutant with this single amino acid substitution is denoted subtilisin S221C. The resulting S221C subtilisin mutant is designated S1.

The subtilisin may be further mutated in order to make the relatively unstable apoenzyme easier to produce and purify. Versions of S1 with three or four additional mutations, for example, M50F, Q206I, Y217K and N218S, may also be employed in the method of the present invention. Such further mutations

---

[1] The S221A mutant was originally constructed for this purpose. The mature form of this mutant was heterogeneous on its N-terminus, however, presumably due to some incorrect processing of the pro-enzyme.

cumulatively increase the free energy of unfolding by 3.0 kcal/mol and increase the thermal denaturation temperature of the apoenzyme by 11.5°C (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). The mutant containing the M50F, Q206I, Y217K, N218S and S221C mutations is designated S11 and the mutant containing the M50F, Y217K, N218S and S221C is designated S12.[2]

In order to produce a subtilisin BPN' protein lacking calcium binding activity, the present inventors elected to delete the binding loop in the calcium A-site to engineer a novel calcium-free subtilisin protein. This loop comprises an interruption in the subtilisin BPN' α-helix involving amino acids 63-85 of SEQUENCE ID NO:1 (McPhalen and James 1988). Residues 75-83 of the subtilisin BPN' protein form a loop which interrupts the last turn of the 14-residue alpha helix involving amino acids 63-85 [SEQUENCE ID NO:1].[3] Four of the carbonyl oxygen ligands to the calcium are provided by a loop composed of amino acids 75-83 [SEQUENCE ID NO:1]. The geometry of the ligands is that of a pentagonal bipyramid whose axis runs through the carbonyls of amino acids 75 and 79. On one side of the loop is the bidentate carboxylate (D41), while on the other side is the N-terminus of the protein and the side chain of Q2. The seven coordination distances range from 2.3 to 2.6 A, the shortest being to the aspartyl carboxylate. Three hydrogen bonds link the N-terminal segment to loop residues 78-82 in parallel-beta arrangement. A high affinity calcium binding site is a common feature of subtilisins which make large contributions to their high stability. By binding at specific sites in the subtilisin tertiary structure,

---

[2] The specific activities of S11, S12 and S15 against the synthetic substrate, SAAPFna, are the same. (S.A. = 0.0024 U/mg at 25°C, pH 8.0). These measurements were performed on protein freshly purified on a mercury affinity column.

[3] This set of nine residues was chosen for deletion, as opposed to 74-82 (those actually belonging to the loop) out of preference for Ala 74 rather than Gly 83 in the resulting continuous helix. Alanine has a higher statistical likelihood for occurrence in α-helix, due to glycine's broader range of accessible backbone conformations.

tertiary structure, calcium contributes its binding free energy to the stability of the native state. In the present invention, site-directed mutagenesis was used to delete amino acids 75-83 of SEQUENCE ID NO:1, which creates an uninterrupted helix and abolishes the calcium binding potential at site A (Figure 1A and 1B).

The present inventors believed that a stabilization strategy based around calcium binding would allow survival in the extracellular environment. Since the major industrial uses of subtilisins are in environments containing high concentrations of metal chelators, it was of great practical significance for the present inventors to produce a stable subtilisin which is independent of calcium and, therefore, unaffected by the presence of metal chelating agents.

While the present inventors chose to eliminate calcium binding by the removal of these amino acids, it should be possible to eliminate calcium binding by other mutations, e.g., substitution of one or more of the amino acids at positions 75-83 with alternative amino acids and by insertion, substitution and/or deletion of amino acids proximate to positions 75-83. This may also be accomplished by site-specific mutagenesis.

Additionally, because this loop is a common feature of subtilisins, it is expected that equivalent mutations for other subtilisins, in particular class I subtilases, e.g., by site-specific mutagenesis, will likewise eliminate calcium binding and provide for enzymatically active mutants.

In particular, the present inventors synthesized by site-specific mutagenesis three subtilisin BPN' DNA's which have been mutated to eliminate amino acids 75-83 involved in calcium binding and which further comprise additional substitution mutations. These mutated subtilisin BPN' DNA's, upon expression of the DNA, provide for subtilisin proteins having enhanced thermal stability and/or which are resistant to proteolysis.

The specific subtilisin BPN' mutants synthesized by the present inventors are designated in this application as S15, S39, S46, S47, S68, S73, S79, and S86. The specific point mutations set forth in the present application identify the

particular amino acids in the subtilisin BPN' amino acid sequence, as set forth in SEQUENCE ID NO:1, that are mutated in accordance with the present invention. For example, the S15 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: S221C, N218S, M50F and Y217K. The S39 mutant similarly comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: S221C, P5A, N218S, M50F and Y217K.. The S46 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: M50F, Y217K and N218S. The S47 mutant similarly comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: P5A, N218S, M50F and Y217K. The S68 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: P5S, N218S, M50F and Y217K. The S73 mutant comprises a deletion of amino acids 75-83 as well as the following substitution mutations: Q2K, M50F, A73L, Q206V, Y217K and N218S. The S79 mutant comprises a deletion of amino acids 75-83 and additional comprises the following substitution mutations: Q2K, M50F, A73L, Q206C, Y217K and N218S. Finally, the S86 mutant comprises a deletion of amino acids 75-83 as well as the following substitution mutations: Q2K, S3C, M50F, A73L, Q206C, Y217K and N218S. The specific activities of the proteolytically active S46, S47, S68, S73, S79 and S86 subtilisins have been found to be similar or enhanced in relation to the wild-type enzyme.

The various Δ75-83 subtilisins which were synthesized by the inventors are shown in Table I, below. The particular points of mutation in the amino acid sequence of subtilisin BPN' amino acid sequence, as set forth in SEQUENCE ID NO:1, are identified. The synthesis of these mutants is described in more detail *infra*.

TABLE I: Subtilisin Mutations

	S221C	P5A	Δ75-83	N218S	M50P	Q206I	Y217K	Q271E	Q2K	A73L	Q206V	Q206C	S3C
BPN'	-	-	-	-	-	-	-	-	-	-	-	-	-
S1*	+	-	-	-	-	-	-	-	-	-	-	-	-
S11*	+	-	-	+	+	+	+	-	-	-	-	-	-
S12*	+	-	-	+	+	-	+	-	-	-	-	-	-
S15*	+	-	+	+	+	-	+	-	-	-	-	-	-
S39	+	+	+	+	+	-	+	-	-	-	-	-	-
S46	-	-	+	+	+	-	+	-	-	-	-	-	-
S47	-	+	+	+	+	-	+	-	-	-	-	-	-
S68	-	P5S	+	+	+	-	+	-	-	-	-	-	-
S73	-	-	+	+	+	-	+	+	+	+	+	-	-
S79	-	-	+	+	+	-	+	+	+	+	-	+	-
S86	-	-	+	+	+	-	+	+	+	+	-	+	+

The plus signs show that a subtilisin contains a particular mutation. X-ray crystal structures of wild type,S12 and S15 have been determined to 1.8Å.

*S1, S11, S12, S15 and S39 are low activity mutants constructed to aid in the evaluation of structure and conformational stability.

In order to understand the contribution of calcium binding to the stability of subtilisin, the thermodynamics and kinetics of calcium binding to the high affinity calcium A-site were measured by microcalorimetry and fluorescence spectroscopy. Calcium binding is an enthalpically driven process with an association constant (K_a) equal to 7×10^6 M^{-1}. The kinetic barrier to calcium removal from the A-site (23 kcal/mol) is substantially larger than the standard free energy of binding (9.3 kcal/mol). The kinetics of calcium dissociation from subtilisin (e.g., in excess EDTA) are accordingly very slow. For example, the half-life ($t_{1/2}$) of calcium dissociation from subtilisin, i.e., the time for half of the calcium to dissociate from subtilisin, is 1.3 hours at 25°C.

X-ray crystallography shows that except for the region of the deleted calcium-binding loop, the structure of the subtilisin mutants and the wild type protein are very similar. The N-terminus of the wild-type protein lies beside the site A loop and furnishes one calcium coordination ligand, the side chain oxygen of Q2. In Δ75-83 subtilisin, the loop is gone, leaving residues 1-4 disordered. These first four residues are disordered in the X-ray structure since all its interactions were with the calcium loop. N-terminal sequencing confirms that the first four amino acids are present, confirming that processing occurs at the normal site. The helix is shown to be uninterrupted and shows normal helical geometry over its entire length. X-ray crystallography further shows that the structures of subtilisin with and without the deletion superimpose with a root mean square (r.m.s.) difference between 261 α-carbon positions of 0.17 Å, and are remarkably similar considering the size of the deletion. Diffuse difference density and higher temperature factors, however, indicate some disorder in the newly exposed residues adjacent to the deletion.

While the elimination of calcium binding is advantageous since it produces proteins that are more stable in the presence of metal chelators, it is disadvantageous in at least one respect. Specifically, the elimination of the calcium loop without any other compensating mutations results in the destabilization of the native state relative to the partially folded states and,

therefore, a loss of cooperativity in folding. The present inventors thus sought to further genetically engineer the subtilisin S15 BPN' protein lacking amino acids 75-83 in order to restore cooperativity to the folding reaction. In most well designed proteins all parts of the molecule are interdependent, making the unfolding reaction highly cooperative. Cooperativity of the folding reaction allows proteins to achieve sufficient stabilities of the native state for proper function since the overall stability of the native conformation is roughly the sum of all local interactions.

Therefore, while the Δ75-83 subtilisin is an example of an engineered subtilisin which is active and stable in the absence of calcium, the present inventors sought to improve this protein by further mutation. The design of a particular highly stable calcium-free subtilisin relies on an iterative engineering cycle. The present inventors found that the requisite first step in the cycle was to greatly diminish the proteolytic activity of subtilisin. This is necessary because calcium contributes greatly to the conformational stability of subtilisin and the early versions of calcium-free subtilisin are susceptible to proteolysis. After reducing the susceptibility to proteolysis, the next step in the cycle was to eliminate sequences essential for calcium binding, i.e., the A-site. Although the S15 Δ75-83 subtilisin is much less stable than the wild type subtilisin in the presence of calcium, this mutant is more stable than wild type subtilisin in the presence of the metal chelator EDTA.

Accordingly, the third step was to improve the stability of the calcium-free subtilisin protein. To improve the stability of calcium-free subtilisin, the present inventors next tried to create a home for the disordered N-terminal residues. In order to create a highly stable calcium-free subtilisin, the N-terminal part of the protein which is destabilized by the deletion of the calcium A-loop may be modified. For example, the N-terminus which is disordered may be deleted or extended. This, however, is problematic because the requirements for processing the propeptide from the mature protein are not known. It is known, however, that the processing site is not determined by amino acid

sequence since mutants Y1A (the C-terminus of the propeptide), A1C and Q2R do not alter the site of cleavage. It is also known that the native structure of the N-terminus in subtilisin does not determine the cleavage site because the Δ75-83 variants are processed correctly. Since it is not yet known how to alter the processing site, interactions with the existing N-terminus may be optimized.

Examination of the structure of S15 subtilisin revealed numerous possibilities for improving stability of the mutant enzyme. The regions of the structure most affected by the deletion are the N-terminal amino acids 1-8, the 36-45 ω-loop, the 70-74 α-helix the 84-89 helix turn and the 202-219 ß-ribbon. As previously stated, the first four residues in Δ75-83 subtilisin are disordered in the x-ray structure since all its interactions had been with the calcium loop. N-terminal sequencing shows, however, that the first four amino acids are present confirming that processing occurs at the normal site. Other than the N-terminus, there are three other residues whose side chain conformations are distinctly different from wild type. Y6 swings out of a surface niche into a more solvent-exposed position, as an indirect effect of the destabilization of the N-terminus. D41, a former calcium ligand, and Y214 undergo a coordinated rearrangement, forming a new hydrogen bond. The B-factors of all three residues increase significantly due to the deletion of amino acids 75-83. In addition, S87 and A88 do not change conformation but exhibit significantly increased B-factors. P86 terminates the α-helix from which the calcium loop was deleted. In view of the above, other mutations at one or more of the above mentioned sites, or at the amino acids proximate thereto, will provide for subtilisin BPN′ mutants comprising greater enzymatic activity or increased stability.

There are several logical strategies for remodeling this region of the protein to produce subtilisin BPN′ mutants comprising greater enzymatic activity or increased stability. Since the N-terminal four amino acids are disordered in the x-ray structure, one possible approach would be to delete them from the protein. The requirements for processing the propeptide from the mature protein

are not understood, however. Inserting or deleting amino acids from the N-terminal region is, therefore, problematic. For this reason insertions and deletions in the N-terminal region were avoided in favor of amino acid substitutions. Many of the original amino acids in the above described regions of subtilisin which interacted with the amino acids 75-83 loop can be assumed to no longer be optimal. It was, therefore, possible to increase the stability of the molecule by substituting, deleting or adding at least one amino acid at positions whose environment was changed by the 75-83 deletion.

The first attempt was to mutate the proline at position 5 to alanine to create more flexibility at position 5. This increased flexibility allows the N-terminus to try to find a unique position along the new surface of the protein, created by deletion of the calcium loop. Once the N-terminus assumes a unique location its local interactions may then be optimized.

The P5A mutation was made to try to create more flexibility for the N-terminus and allow it to find a unique position along the new surface of the protein that was created by deletion of the calcium loop. In the native structure, the first five amino acids are in an extended conformation and form ß-pair hydrogen bonds with the calcium loop as well as the Q2 side chain interaction with the calcium. The proline at position 5, which is conserved among seven bacterial subtilisins which have a homologous calcium A-site, may help stabilize the extended conformation. The P5A mutation in Δ75-83 subtilisin should thus result in an increase in the cooperativity of the unfolding reaction. The X-ray structure of this variant has been determined to 1.8 Å.

In *toto*, the present inventors selected amino acids at ten different positions whose environment had changed substantially for substitution. A mutagenesis and screening procedure was developed in order to screen all possible substitutions at a particular site. The technique for generating and screening subtilisin variants involves *in vitro* mutagenesis of the cloned subtilisin gene, expression of the mutated genes in *B. subtilis*, and screening for enhanced stability.

For example, site-directed mutagenesis was performed on the S46 subtilisin gene using oligonucleotides which were degenerate at one codon. The degenerate codon contained all combinations of the sequence NNB, where N is any of the four nucleotides and B is T, C or G. The 48 codons represented in this population encode for all twenty amino acids but exclude the ochre and umber termination codons. The mutagenized genes were used to transform *B. subtilis*. Examples of particular mutations are shown in Table II as follows:

Table II - Site-directed mutagenesis

Region of protein	Site	Stabilizing mutations	Mutagenic Oligonucleotide
N-terminus:	Q2	K, W, L	AC GCG TAC GCG NNB TCC GTG CCT TAC
	S3	C*	GCG TAC GCG AAG NNB GTG CCT TAC GG
	V4	none	C GCG AAG TCC NNB CCT TAC GGC G
	P5	S	CAG TCC GTG NNB TAC GGC GTA TC
36-44 omega loop:	D41	A	GAT TCT TCT CAT CCT NNB TTA AAG GTA GC
	K43	R,N	CAT CCT GAT TTA NNB GTA GCA GGC GG
63 -85 α-helix:	A73	L,Q	GGC ACA GTT NNB GCT GTT GCG
	A74	none	C ACA GTT GCG NNB GTT GCG CCA AG
202-220 ß-ribbon:	Q206	I, V, W, C*	C GTA TCT ATC NNB AGC ACG CTT CC
	Y214	none	CCT GGA AAC AAA NTN GGG GCG AAA TC

*Double cysteine mutations at positions 3 and 206 have been found to be as stabilizing as a disulfide bond.

To have a 98% chance of finding tryptophan, glutamine, glutamate or methionine in the mutant population, one must screen about 200 mutant clones. Each of those codons is represented by only one of the 48 codons contained in the population of sequences NNB. Codons for all other amino acids are represented by at least two codons in the population and would require screening of about 100 mutant clones to have a 98% chance of being represented in the mutant population.

To identify the optimum amino acid at a position, mutants were screened for retention of enzymatic activity at high temperature. 100 μl of media was dispensed in each of the 96 wells of a microtiter dish. Each well was inoculated with a *Bacillus* transformant and incubated at 37° with shaking. After 18 hours of growth, 20 μl of culture was diluted into 80 μl of 100 mM Tris-HCl, pH 8.0 in a second microtiter dish. This dish was then incubated for one hour at 65°C. The dish was allowed to cool to room temperature following the high temperature incubation and 100 μl of 1 mM SAAPF-pNA was added to each well. The wells which cleaved the pNA (turned yellow) quickest were deemed to contain the most heat resistant subtilisin mutant. Once preliminary identification of a stable mutant was made from the second microliter dish, the *Bacillus* clone in the corresponding well in the first microliter dish was grown up for further analysis.

The screening procedure identified stabilizing mutations at seven of the ten positions which were examined. As noted, these amino acid positions were selected at positions of the protein whose environment has changed substantially by virtue of the calcium domain deletion. No mutations were identified at positions 4, 74 and 214 which by themselves significantly increased the half-life of the mutant relative to the parent subtilisin. However, at position 214 the effect of only hydrophobic amino acids was screened. No mutations were found at positions 5, 41 and 43 which resulted in measurable but modest increases in stability. Moreover, several mutations were found at positions at 2, 3, 73, and

206 which significantly increased the half-life of the mutant relative to the parent subtilisin. These stabilizing mutations are shown in Table III as follows:

Table III - Stabilizing Mutations

Region of protein	Site	Increase
N-terminus:	Q2K	2.0-fold
63-85 α-helix:	A73L	2.6-fold
202-220 β-ribbon	Q206V	4.5-fold
N-terminus - β-ribbon	S3C-Q206C (disulfide)	14-fold

Stabilizing amino acid modifications at positions 2(K), 73(L) and 206(V) were then combined to create subtilisin S73. The properties of S73 subtilisin as well as S46, S79 and S86 are summarized in Table IV.

Table IV

Mutant	Mutations[1]	Specific activity[2]	Half-life (60°C)[3]	Increase
S46	-	100U/mg	2.3 min	-
S73	Q2K A73L Q206V	160U/mg	25 min	11-fold
S79	Q2K A73L Q206C	N.D.[4]	18 min	8-fold
S86	Q2K S3C[5] A73L Q206C[5]	85U/mg	80 min	35-fold

1) All of subtilisins S46, S73, S79 and S86 contain the mutations M50F, Y217K and N218S and Q271E.
2) Specific activity is measured against succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide(SAAPF-pNA) in 10mM Tris-HCl, pH 8.0 at 25°C.
3) Half-life is measured at 60°C in 10mM Tris-HCl, pH 8.0 50mM NaCl and 10mM EDTA.
4) Not determined.
5) Disulfide bond was formed between the cysteines at positions 3 and 206. The formation of a disulfide bond was confirmed by measuring the radius of gyration of the denatured protein by gel electrophoresis.

In many cases, the choice of amino acid at a particular position will be influenced by the amino acids at neighboring positions. Therefore, in order to find the best combinations of stabilizing amino acids, it will be necessary in some cases to vary the amino acids at two or more positions simultaneously. In particular, this was effected at positions 3 and 206 with amino acids whose side chains can potentially interact. It was determined that the best combination of modifications was cysteine modifications at positions 3 and 206. This modification was denoted as S86. Because of the close proximity and suitable geometry between the cysteines at these two positions a disulfide cross-link forms spontaneously between these two residues.

The stability of the S86 subtilisin was studied in relation to S73. It was found that the half-life of S86 is 80 minutes at 60°C in 10mM Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA, a 3.2-fold enhancement relative to S73

subtilisin. By contrast, a 3-206 disulfide cross-link would not be able to form in native subtilisins which contain the calcium A-site because the 75-83 binding loop separates the N-terminal amino acids from the 202-219 ß ribbon. Therefore, the enhancement in stability which occurs in the subject S86 mutant lacking the 75-83 binding loop will likely not be observed with native subtilisins similarly cysteine modified at these positions.

It is expected that similar enhancement in stability will be inherent to other subtilisins of the I-S1 and I-S2 group if their calcium loops were deleted (see Siezen et al, Protein Engineering, 4, pp. 719-737 at Figure 7). This is a reasonable expectation based on the fact that the primary calcium site in these different subtilisins are formed from almost identical 9 residue loops comprised in the identical position of helix C.

X-ray structures of the I-S1 subtilisins BPN and Carlsberg, as well as the I-S2 subtilisin (savinase), have been determined to high resolution. Comparison of these structures demonstrates that all three have almost identical calcium A-sites.

The x-ray structure of the class I subtilase, thermitase from *Thermoactinomyces vulgaris*, is also known. Though the overall homology of BPN′ to thermitase is much lower than the homology of BPN′ to I-S1 and I-S2 subtilisins, thermitase has been shown to have an analogous calcium A-site. In the case of thermitase, the loop is a ten residue interruption at the identical site in helix C.

Thus, it is expected that the stabilizing mutations exemplified herein will impart similar beneficial effects on stability for the calcium loop-deleted versions of other class I subtilases.

The stability of S73, S76 and S86 subtilisins relative to S46 subtilisin was compared by measuring their resistance to thermal inactivation at 60°C in 10mM Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA. Aliquots were removed at intervals and the activity remaining in each aliquot was determined. Under these

conditions, the half-life of S46 subtilisin is 2.3 minutes and the half-life of S73 is 25 minutes (Table IV).

In order to identify other mutants having increased stability any mutagenesis technique known by those skilled in the art may be used. One example of such a technique for generating and screening subtilisin variants involves three steps: 1) *in vitro* mutagenesis of the cloned subtilisin gene; 2) expression of mutated genes in *B. subtilis*, and 3) screening for enhanced stability. The key element in the random mutagenesis approach is being able to screen large numbers of variants.

Although random mutagenesis may be employed, the mutagenesis procedure described above allows for mutations to be directed to localized regions of the protein (e.g., the N-terminal region). As noted supra, the S46, S47, S68, S73, S79 and S86 mutants (which comprise the active-site S221) were found to be enzymatically active. It is expected that other substitutions may be identified which provide for equivalent or even greater stability and activity.

The activities of examples of the calcium-free subtilisin mutants of the present invention against the substrate sAAPF-pNA in Tris-HCl, pH 8.0 and 25°C are given in Table V as follows:

Table V

Subtilisin	Specific activity	Half-life (55°C)
BPN'	80 U/mg	2 min
S12	0.0025 U/mg	N.D.[1]
S15	0.0025 U/mg	N.D.[1]
S39	0.0025 U/mg	N.D.[1]
S46	125 U/mg	22 min
S47	90 U/mg	4.7 min
S68	~100 U/mg	25 min

1) Half-lives were not determined for inactive subtilisins.

As shown above, the subtilisin mutants S46, S47, S68, S73, S79 and S86 have enhanced catalytic activity in comparison with subtilisin BPN'. Changes in catalytic efficiency due to the deletion were not expected because of the fact that the active site of subtilisin is spatially distant from the calcium A-site.

The stability of these mutant subtilisins was compared to native subtilisin BPN' by measuring their resistance to thermal inactivation. Since the stability of the calcium-free subtilisin mutants should be unaffected by metal chelating agents, the experiment was carried out in 10mM Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA (the association constant of EDTA for calcium is 2×10^8 M^{-1}). The proteins were dissolved in this buffer and heated to 55°C. Aliquots were removed at intervals and the activity remaining in each aliquot was determined. The kinetics of inactivation are plotted in Figure 10. Under these conditions, the half-life of the subtilisin mutants was much improved over that of subtilisin BPN'. These results indicate that subtilisins which have been mutated to eliminate calcium binding at site A have full catalytic activity and improved stability in EDTA relative to subtilisin BPN'. A reasonable level of stability in S46 was achieved even without additional mutations to compensate for lost interactions resulting from deleting amino acids 75-83.

Thus, the present inventors have provided convincing evidence that subtilisin mutants may be obtained which remain active and yet do not bind calcium. It is expected therefore that these mutants may be utilized in industrial environments that comprise chelating agents. While this has only been specifically shown with subtilisin BPN', equivalent mutations should work with other serine proteases as well, most particularly other I-S1 or I-S2 subtilisins given that these subtilisins possess substantial sequence similarity, especially in the calcium binding site.

Such strategies, for example, may involve comparing the sequence of subtilisin BPN' to other serine proteases in order to identify the amino acids which are suspected to be necessary for calcium binding and then making suitable modifications, e.g., by site-specific mutagenesis. Since many subtilisins are

related to subtilisin BPN' not only through their primary sequences and enzymological properties, but also by X-ray crystallographic data, it is expected that other active subtilisin mutants which lack calcium binding may be produced by site specific mutagenesis. For example, structural evidence exists that the homologous enzyme subtilisin Carlsberg also comprises two calcium binding sites. Similarly, the X-ray structure of thermitase is known and this subtilisin has an analogous calcium A binding-site to that of subtilisin BPN'. For thermitase, the calcium binding loop is a ten residue interruption at the identical site in helix C. Accordingly, these enzymes should also be amenable to the mutations described herein which eliminate the calcium binding site and produce a stable, active enzyme. Moreover, as discussed supra, Siezen et al, has demonstrated that the primary calcium binding site in all subtilisins in groups I-S1 and I-S2 are formed from almost identical nine residue loops in the identical position of helix C. Thus, in view of the almost identical structures of the calcium A-sites, the methods described herein should be applicable to most if not all of the subtilisins in groups I-S1 and I-S2 set forth in Siezen et al.

Alternatively, if the amino acids which comprise the calcium binding sites are already known for a particular subtilisin, the corresponding DNA will be mutated by site specific mutagenesis to delete one or more of such amino acids, or to provide substitution, deletion or addition mutations which eliminate calcium binding.

The subject mutant subtilisins will generally be produced by recombinant methods, in particular by expression of a subtilisin DNA which has been mutated such that upon expression it results in a subtilisin protein which is enzymatically active and which does not bind calcium.

Preferably, the subtilisin DNA's will be expressed in microbial host cells, in particular *Bacillus subtilis*, because this bacteria naturally produces subtilisin, is an efficient secretor of proteins, and is able to produce the protein in an active conformation. However, the invention is not restricted to the expression of the subtilisin mutant in *Bacillus*, but rather embraces expression in any host cell

which provides for expression of the desired subtilisin mutants. Suitable host cells for expression are well known in the art and include, e.g., bacterial host cells such as *Escherichia coli, Bacillus, Salmonella, Pseudomonas*; yeast cells such as *Saccharomyces cerevisiae, Pichia pastoris, Kluveromyces, Candida, Schizosaccharomyces*; and mammalian host cells such as CHO cells. Bacterial host cells, however, are the preferred host cells for expression.

Expression of the subtilisin DNA will be provided using available vectors and regulatory sequences. The actual selection will depend in large part upon the particular host cells which are utilized for expression. For example, if the subtilisin mutant DNA is expressed in *Bacillus*, a *Bacillus* promoter will generally be utilized as well as a *Bacillus* derived vector. The present inventors in particular used the pUB110-based expression vector and the native promoter from the subtilisin BPN' gene to control expression on *Bacillus subtilis*.

It is further noted that once the amino acid sequence of a particular subtilisin mutant which does not bind calcium has been elucidated, it may also be possible to make the subtilisin mutant by protein synthesis, e.g., by Merrifield synthesis. However, expression of the subtilisin mutants in microbial host cells will generally be preferred since this will allow for the microbial host cell to produce the subtilisin protein in a proper conformation for enzymatic activity. However, since the present inventors further teach herein a method for obtaining *in vitro* refolding of the subtilisin mutant, it should be possible to convert improperly folded subtilisin mutants into an active conformation.

In order to further illustrate the present invention and the advantages thereof, the following specific examples are given, it being understood that the same is intended only as illustrative and in nowise limitative.

EXAMPLES

Example 1

Cloning and Expression The subtilisin gene from *Bacillus amyloliquefaciens* (subtilisin BPN') has been cloned, sequenced, and expressed at high levels from its natural promoter sequences in *Bacillus subtilis* (Wells et al., Nucleic Acids Res. 11:7911-7925 (1983); Vasantha et al., J. Bacteriol. 159:811-819 (1984)). All mutant genes were recloned into a pUB110-based expression plasmid and used to transform *B. subtilis*. The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild type (wt) activity (Fahnestock et al., Appl. Environ. Microbial. 53:379-384 (1987)). Oligonucleotide mutagenesis was carried out as previously described. (Zoller et al., Methods Enzymol. 100:468-500 (1983); Bryan et al., Proc. Natl. Acad. Sci. 83:3743-3745 (1986b)). S221C was expressed in a 1.5l New Brunswick fermentor at a level of approximately 100 mg of the correctly processed mature form per liter. The addition of wild type subtilisin to promote production of the mature form of S221C subtilisin was not required in our bacillus host strain as was the case for prior strains (Abrahmsen et al., Biochemistry 30:4151-4159 (1991)).

Protein Purification & Characterization. Wild type subtilisin and the variant enzymes were purified and verified for homogeneity essentially as described in Bryan et al., Proc. Natl. Acad. Sci. 83:3743-3745 (1986b); Pantoliano et al., Biochemistry 26:2077-2082 (1987); and Biochemistry 27:8311-8317 (1988). In some cases the C221 mutant subtilisins were re-purified on a sulfhydryl specific mercury affinity column (Affi-gel 501, Biorad). Assays of peptidase activity were performed by monitoring the hydrolysis of succinyl-(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-p-nitroanilide, hereinafter sAAPFna, as described by DelMar et al., Anal Biochem. 99:316-320 (1979). The protein concentration, [P], was determined using $P^{0.1\%} = 1.17$ at 280 nm (Pantoliano et al, Biochemistry 28:7205-7213 (1989)). For variants which contain the Y217K change, the $P^{0.1\%}$ at 280 nm was calculated to be 1.15 (or 0.96 X wt), based on

the loss of one Tyr residue (Pantoliano et al., Biochemistry 28:7205-7213 (1989)).

N-terminal Analysis The first five amino acids of subtilisin S15 were determined by sequential Edman degradation and HPLC analysis. This revealed that 100% of the material had the amino acid sequence expected from the DNA sequence of the gene and that processing of the pro-peptide was at the same position in the sequence for the mutant as for the wild type enzyme.

Example 2

Structure of the calcium A site of S12 subtilisin Calcium at site A is coordinated by five carbonyl oxygen ligands and one aspartic acid. Four of the carbonyl oxygen ligands to the calcium are provided by a loop composed of amino acids 75-83 (Figure 2). The geometry of the ligands is that of a pentagonal bipyramid whose axis runs through the carbonyls of 75 and 79. On one side of the loop is the bidentate carboxylate (D41), while on the other side is the N-terminus of the protein and the side chain of Q2. The seven coordination distances range from 2.3 to 2.6 Å, the shortest being to the aspartyl carboxylate. Three hydrogen bonds link the N-terminal segment to loop residues 78-82 in parallel-beta arrangement.

Preparation of apo-subtilisin S11 and S12 subtilisin contain an equal molar amount of tightly bound calcium after purification. X-ray crystallography has shown this calcium to be bound to the A site (Finzel et al., J. Cell. Biochem. Suppl. 10A:272 (1986); Pantoliano et al., Biochemistry 27:8311-8317 (1988); McPhalen et al., Biochemistry 27:6582-6598 (1988)).

Complete removal of calcium from subtilisin is very slow, requiring 24 hours of dialysis against EDTA at 25°C to remove all calcium from the protein and then 48 more hours of dialysis in high salt (Brown et al., Biochemistry 16:3883-3896 (1977)) at 4°C to remove all EDTA from the protein. To prepare the calcium-free form of subtilisins S11 and S12, 20mg of lyophilized protein was dissolved in 5ml of 10mM EDTA, 10mM tris(hydroxymethyl)amino-methane

hydrochloric acid (hereinafter Tris-HCl) at pH 7.5 and dialyzed against the same buffer for 24 hours at 25°C. In order to remove EDTA, which binds to subtilisin at low ionic strength, the protein was then dialyzed twice against 2 liters of 0.9M NaCl, 10mM Tris-HCl at pH 7.5 at 4°C for a total of 24 hours and then three times against 2 liters of 2.5mM Tris-HCl at pH 7.5 at 4°C for a total of 24 hours. Chelex 100 was added to all buffers not containing EDTA. When versions of C221 subtilisin not containing stabilizing amino acid substitutions were used, up to 50% of the protein precipitated during this procedure. It is essential to use pure native apoenzyme in titration experiments so that spurious heat produced by precipitation upon the addition of calcium does not interfere with the measurement of the heat of binding.

To ensure that preparations of apo-subtilisin were not contaminated with calcium or EDTA, samples were checked by titration with calcium in the presence of Quin2 prior to performing titration calorimetry.

Titration Calorimetry Measurements The calorimetric titrations were performed with a Microcal Omega titration calorimeter as described in detail by Wiseman et al., Analytical Biochemistry 179:131-137 (1989). The titration calorimeter consists of a matched reference cell containing the buffer and a solution cell (1.374 mL) containing the protein solution. Microliter aliquots of the ligand solution are added to the solution cell through a rotating stirrer syringe operated with a plunger driven by a stepping motor. After a stable baseline was achieved at a given temperature, the automated injections were initiated and the accompanying heat change per injection was determined by a thermocouple sensor between the cells. During each injection, a sharp exothermic peak appeared which returned to the baseline prior to the next injection occurring 4 minutes later. The area of each peak represents the amount of heat accompanying binding of the added ligand to the protein. The total heat (Q) was then fit by a nonlinear least squares minimization method (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)) to the total ligand concentration, $[Ca]_{total}$, according to the equation:

$$dQ/d[Ca]_{total} = \Delta H[1/2+(1-(1+r)/2-Xr/2)/Xr-2Xr(1-r)+1+r^2)^{1/2}] \quad (1)$$

wherein $1/r = [P]_{total} x K_a$ and $X_r = [Ca]_{total}/[P]_{total}$.

The binding of calcium to the S11 and S12 subtilisins was measured by titration calorimetry as it allows both the binding constant and the enthalpy of binding to be determined (Wiseman et al., Analytical Biochemistry 179:131-137 (1989); Schwarz et al., J. Biol. Chem. 266:24344-24350 (1991)).

The S11 and S12 subtilisin mutants were used in titration experiments because production of the wild type apoenzyme is impossible due to its proteolytic activity and low stability. Titrations of S11 and S12 were performed at protein concentrations [P] = 30μM and 100μM. Titration of the S11 apoenzyme with calcium at 25°C is shown in Figure 4. The data points correspond to the negative heat of calcium binding associated with each titration of calcium. The titration calorimeter is sensitive to changes in K_a under conditions at which the product of K_a x [P] is between 1 and 1000 (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)). Since the K_a for subtilisin is about $1x10^7$ M^{-1}, these protein concentrations result in values of K_a x [P] = 300 and 1000. At lower protein concentrations the amount of heat produced per titration is difficult to measure accurately.

The results of fitting the titrations of S11 and S12 to a calculated curve are summarized in Table 2. The parameters in the table include binding parameters for stoichiometric ratio (n), binding constant (K_a) and binding enthalpy (ΔH). These parameters were determined from deconvolution using nonlinear least squares minimization (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)). Measurements for each experimental condition were performed in duplicate at 25°C. The protein concentrations ranged from 30 to 100 μM while the concentration of the calcium solutions were about 20 times the protein concentrations. Each binding constant and enthalpy were based on several titration runs at different concentrations. Titration runs were performed until the titration peaks were close to the baseline.

TABLE 2: Titration Calorimetry of the Calcium A Site in Subtilisin Mutants S11 and S12.

Mutant	[P]	*Parameters calculated from fit*		
		n	K_a	ΔH
S11	100μM	0.98 ± 0.01	7.8 ± 0.2 x 10^6	-11.3 ± 0.1
S11	33μM	0.9 ± 0.3	6.8 ± 1.5 x 10^6	-10.9 ± 0.2
S12	100μM	0.99 ± 0.01	6.4 ± 0.2 x 10^6	-11.8 ± 0.5

The average values obtained are similar for S11 and S12: ΔH = ~ -11 kcal/mol; K_a = 7 x 10^6 M^{-1} and a stoichiometry of binding of 1 calcium site per molecule. The weak binding site B does not bind calcium at concentrations below the millimolar range, and therefore does not interfere with measurement of binding to the binding site A. The standard free energy of binding at 25°C is 9.3 kcal/mol. The binding of calcium is therefore primarily enthalpically driven with only a small net loss in entropy ($\Delta S_{binding}$ = -6.7 cal/°mol).

Example 3

In vitro refolding of S15 subtilisin. For refolding studies subtilisin was maintained as a stock solution in 2.5 mM Tris-HCl at pH 7.5 and 50mM KCl at a concentration of approximately 100μM. The protein was denatured by diluting the stock solution into 5M guanidine hydrochloride (Gu-HCl) at pH 7.5 or in most cases into 25 mM H_3PO_4 or HCl at pH 1.8 - 2.0. The final concentration of protein was 0.1 to 5 μM. S15 was completely denatured in less than 30 seconds by these conditions. S12 required approximately 60 minutes to become fully denatured. Acid-denatured protein was then neutralized to pH 7.5 by the addition of Tris-base (if denatured in HCl) or 5M NaOH (if denatured in H_3PO_4). Refolding was initiated by the addition of KCl, NaCl or $CaCl_2$ to the

desired concentration. For example, KCl was added from a stock solution of 4M to a final concentration of 0.1 to 1.5M with rapid stirring. In most cases renaturation was carried out at 25°C. The rate of renaturation was determined spectrophotometrically by *uv* absorption from the increase in extinction at λ = 286, from the increase in intrinsic tyrosine and tryptophan fluorescence (excitation λ = 282, emission λ - 347), or by circular dichroism from the increase in negative ellipticity at λ = 222 nm.

Example 4

X-ray Crystallography. Large single crystal growth and X-ray diffraction data collection were performed essentially as previously reported (Bryan et al., Proteins: Struct. Funct. Genet. 1:326-334 (1986a); Pantoliano et al., Biochemistry 27:8311-8317 (1988); Pantoliano et al., Biochemistry 28:7205-7213 (1989)) except that it was not necessary to inactivate the S221C variants with diisopropyl fluorophosphate (DFP) in order to obtain suitable crystals. The starting model for S12 was made from the hyperstable subtilisin mutant 8350 (Protein Data Bank entry 1SO1.pdb). The S12 structure was refined and then modified to provide the starting model for S15.

Data sets with about 20,000 reflections between 8.0 Å and 1.8 Å resolution were used to refine both models using restrained least-squares techniques (Hendrickson et al., "Computing in Crystallography" *in* Diamond et al., eds., Bangalore: Indian Institute of Science 13.01-13.23 (1980)). Initial difference maps for S15, phased by a version of S12 with the entire site A region omitted, clearly showed continuous density representing the uninterrupted helix, permitting an initial S15 model to be constructed and refinement begun. Each mutant was refined from R approximately 0.30 to R approximately 0.18 in about eighty cycles, interspersed with calculations of electron density maps and manual adjustments using the graphics modeling program FRODO (Jones, J. Appl. Crystallogr. 11:268-272 (1978)).

Except for the region of the deleted calcium-binding loop, the structures of S12 and S15 are very similar, with a root mean square (r.m.s) deviation of 0.18 Å between 262 α-carbons. The N-terminus of S12 (as in the wild-type) lies beside the site A loop, furnishing one calcium coordination ligand, the side chain oxygen of Q2. In S15 the loop is gone, leaving residues 1-4 disordered. In S12 (as in wild type) the site A loop occurs as an interruption in the last turn of a 14-residue alpha helix; in S15 this helix is uninterrupted and shows normal helical geometry over its entire length. Diffuse difference density and higher temperature factors indicate some disorder in the newly exposed residues adjacent to the deletion.

Example 5

Differential Scanning Calorimetry The stability properties of S12 and S15 were studied using DSC (differential scanning calorimetry). The Δ75-83 mutant (S15) is very similar in melting temperature to the apoenzyme of S12. The DSC profiles of apo-S12 and S15 are shown in Figure 3. The temperature of maximum heat capacity is 63.0°C for S15 and 63.5°C for apo-S12 at pH 9.63. The DSC experiments were carried out at high pH to avoid aggregation during the denaturation process. The amount of excess heat absorbed by a protein sample as the temperature increased through a transition from the folded to unfolded state at constant pressure, which provided a direct measurement of the ΔH of unfolding (Privalov et al., Methods Enzymol. 131:4-51 (1986)). ΔH_{cal} of unfolding for apo-S12 and S15 is about 140 kcal/mol. Above pH 10.0, the unfolding transition for S15 fit a two-state model reasonably well, consistent with equilibrium thermodynamics as expressed in the van't Hoff equation ($d\ln K / dT = \Delta H_{vH} / (RT^2)$) with ΔH_{vH} (the van't Hoff enthalpy or apparent enthalpy) approximately equal to ΔH_{cal} (the calorimetric or true enthalpy). At pH 9.63, however, the melting profile for both proteins was asymmetric indicating that the unfolding is not a pure two-state process.

Example 6

Measuring kinetics of calcium dissociation. The dissociation of calcium from subtilisin is a slow process. To measure this rate the fluorescent calcium chelator Quin 2 was used. Quin 2 binds calcium with a K_a of 1.8×10^8 at pH 7.5 (Linse et al., Biochemistry 26:6723-6735 (1987)). The fluorescence of Quin 2 at 495nm increases by approximately 6-fold when bound to calcium (Bryant, Biochem. J. 226:613-616 (1985)). Subtilisin S11 or S12 as isolated contains one calcium ion per molecule. When mixed with an excess of Quin 2, the kinetics of calcium release from the protein can be followed from the increase in fluorescence at 495nm. The reaction is assumed to follow the pathway N(Ca) $\Leftrightarrow$ N + Ca + Quin 2 $\Leftrightarrow$ Quin(Ca). The dissociation of calcium from subtilisin is very slow relative to calcium binding by Quin 2, such that the change in fluorescence of Quin 2 is equal to the rate of calcium dissociation from subtilisin. As can be seen in Figure 5a, the initial release of calcium from S11 follows simple first order kinetics.

Temperature dependence of calcium dissociation The first order rate constant (k) for calcium dissociation was measured from 20° to 45°C. The plot of ln k vs. 1/T°K is roughly linear. The calcium dissociation data was curve fit using transition state theory according to the Erying equation:

$$\Delta G^{\ddagger} = -RT \ln K^{\ddagger} = -RT \ln kh/k_B T \qquad (2)$$

wherein k_B is the Boltzman constant, h is Planck's constant and k is the first order rate constant for folding. A graph of ln hk/k_BT vs. 1/T is shown in Figure 5b.

The data was then curve fit according to the equation (Chen et al., Biochemistry 28:691-699 (1989)):

$$\ln K^{\ddagger} = A + B(To/T) + C \ln (To/T) \qquad (3)$$

wherein $A = [\Delta Cp^{\ddagger} + \Delta S^{\ddagger}(To)]/R$; $B = A - \Delta G^{\ddagger}(T_o)/RTo$; $C = \Delta Cp^{\ddagger}/R$. The data obtained yields the following results: $\Delta G^{\ddagger} = 22.7$ kcal/mol; $\Delta Cp'^{\ddagger} = -0.2$ kcal/°mol; $\Delta S'^{\ddagger} = -10$ cal/°mol; and $\Delta H'^{\ddagger} = 19.7$ kcal/mol at a reference temperature of 25°C. A possible slight curvature of the plot would be due to a

change in heat capacity associated with formation of the transition state ($\Delta Cp'^{\ddagger}$ = 0.2 kcal/°mol). ΔCp for protein folding has been shown to be closely correlated with a change in exposure of hydrophobic groups to water (Privalov et al., Adv. Protein Chem. 39:191-234 (1988); Livingstone et al., Biochemistry 30:4237-4244 (1991)). In terms of heat capacity, the transition state therefore appears similar to the native protein. The values for $\Delta S'^{\ddagger}$ and $\Delta H'^{\ddagger}$ obtained from Figure 5b indicate that the transition state is enthalpically less favorable than the calcium bound form with only a small change in entropy.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

All references cited herein are incorporated in their entirety, as if individually incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRYAN, Philip N
ALEXANDER, Patrick
STRAUSBERG, Susan L

(ii) TITLE OF INVENTION: CALCIUM FREE SUBTILISIN MUTANTS

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Burns, Doane, Swecker & Mathis
(B) STREET: P.O. Box 1404
(C) CITY: Alexandria
(D) STATE: Virginia
(E) COUNTRY: United States
(F) ZIP: 22313-1404

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Meuth, Donna M
(B) REGISTRATION NUMBER: 36,607
(C) REFERENCE/DOCKET NUMBER: 028755-021

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (703) 836-6620
(B) TELEFAX: (703) 836-2021

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1868 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 450..1599

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 772..1599

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 450
(D) OTHER INFORMATION: /note= "Amino Acid Val at position 450 is fMet."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TTTTTCCGCA ATTATATCAT TGACAATATC AACATCAATG ATATTCATTA TCATTATTTT      60

TATAAAATGG TTTCACAGCT TTTCTCGGTC AAGAAAGCCA AAGACTGATT TCGCTTACGT     120

TTCCATCAGT CTTCTGTATT CAACAAAAGA TGACATTTAT CCTGTTTTTG GAACAACCCC     180

CAAAAATGGA AACAAACCGT TCGACCCAGG AAACAAGCGA GTGATTGCTC CTGTGTACAT     240

TTACTCATGT CCATCCATCG GTTTTTTCCA TTAAAATTTA AATATTTCGA GTTCCTACGA     300

AACGAAAGAG AGATGATATA CCTAAATAGA AATAAAACAA TCTGAAAAAA ATTGGGTCTA     360

CTAAAATATT ATTCCATACT ATACAATTAA TACACAGAAT AATCTGTCTA TTGGTTATTC     420

TGCAAATGAA AAAAAGGAGA GGATAAAGA GTG AGA GGC AAA AAA GTA TGG ATC       473
                               Val Arg Gly Lys Lys Val Trp Ile
                               -107  -105           .  -100

AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC       521
Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser
                -95                 -90                 -85

ACA TCC TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT       569
Thr Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr
            -80                 -75                 -70

ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG AGC GCC GCT AAG AAG       617
Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys
        -65                 -60                 -55

AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA       665
Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys
    -50                 -45                 -40

TAT GTA GAC GCA GCT TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA       713
Tyr Val Asp Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu
-35                 -30                 -25                 -20

TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA GCA       761
Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala
                -15                 -10                 -5

CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC       809
His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala
            1                   5                   10

CCT GCT CTG CAC TCT CAA GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG       857
Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala
    15                  20                  25

GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA       905
Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala
30                  35                  40                  45

GGC GGA GCC AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC       953
Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn
                50                  55                  60

AAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT CTT AAT AAC      1001
Asn Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn
            65                  70                  75

TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA      1049
Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val
        80                  85                  90
```

```
AAA GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC      1097
Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn
 95                  100                 105

GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC      1145
Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser
110                 115                 120                 125

CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA      1193
Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys
                130                 135                 140

GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC      1241
Ala Val Ala Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly
            145                 150                 155

ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT      1289
Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser
        160                 165                 170

GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC      1337
Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe
    175                 180                 185

TCA AGC GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC      1385
Ser Ser Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile
190                 195                 200                 205

CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT ACG TCA      1433
Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser
                210                 215                 220

ATG GCA TCT CCG CAC GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG      1481
Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys
            225                 230                 235

CAC CCG AAC TGG ACA AAC ACT CAA GTC CGC AGC AGT TTA GAA AAC ACC      1529
His Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr
        240                 245                 250

ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC      1577
Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
    255                 260                 265

GTA CAG GCG GCA GCT CAG TAA A ACATAAAAAA CCGGCCTTGG CCCCGCCGGT       1629
Val Gln Ala Ala Ala Gln  *
270                 275

TTTTTATTAT TTTTCTTCCT CCGCATGTTC AATCCGCTCC ATAATCGACG GATGGCTCCC    1689

TCTGAAAATT TTAACGAGAA ACGGCGGGTT GACCCGGCTC AGTCCCGTAA CGGCCAAGTC    1749

CTGAAACGTC TCAATCGCCG CTTCCCGGTT TCCGGTCAGC TCAATGCCGT AACGGTCGGC    1809

GGCGTTTTCC TGATACCGGG AGACGGCATT CGTAATCGGA TCAGAAGCAA AACTGAGCA     1868
```

WHAT IS CLAIMED IS:

1. An enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises one or more deletion, substitution or addition mutations in at least one of the following regions: the N-terminal amino acids 1-8, the ω-loop amino acids 36-45, the α-helix amino acids 70-74 or the helix turn amino acids 84-89.

2. The subtilisin mutant of claim 1, wherein the amino acids at positions 75-83 are deleted to eliminate the ability of said mutant to bind calcium.

3. The subtilisin mutant of claim 2, wherein the mutant lacking amino acids 75-83 has one or more additional mutations in the amino acid sequence.

4. The subtilisin mutant of claim 3, wherein the mutant has one or more additional mutations in the β-ribbon amino acids 202-219.

5. The subtilisin mutant of claim 3, wherein the subtilisin mutant comprises at least one substitution mutation of N218S, M50F, Y217K, P5S, D41A, K43R, K43N, Q271E, Q2K, Q2W, Q2L, A73L, A73Q, Q206C, Q206V, Q206I, Q206W or S3C.

6. The subtilisin mutant of claim 5, wherein the subtilisin mutant comprises the substitution mutations of N218S, M50F, Y217K and P5S.

7. The subtilisin mutant of claim 5, wherein the subtilisin mutant comprises the substitution mutations of N218S, M50F, Y217K, Q271E, Q2K, A73L and Q206V.

8. The subtilisin mutant of claim 5, wherein the subtilisin mutant comprises the substitution mutations of N218S, M50F, Y217K, Q271E, Q2K, A73L and Q206C.

9. The subtilisin mutant of claim 5, wherein the subtilisin mutant comprises the substitution mutations of N218S, M50F, Y217K, Q271E, Q2K, A73L, Q206C and S3C.

10. The subtilisin mutant of claim 1, wherein the subtilisin is from a *Bacillus* strain.

11. The subtilisin mutant of claim 10, wherein the subtilisin mutant is a subtilisin BPN′ mutant, a subtilisin Carlsberg mutant, a subtilisin DY mutant, a subtilisin amylosacchariticus mutant or a subtilisin mesenticopeptidase mutant or a subtilisin Savinase mutant.

12. The subtilisin mutant of claim 11, wherein the subtilisin mutant is a subtilisin BPN′ mutant.

13. A recombinant method which provides for the expression of an enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises one or more deletion, substitution or addition mutations in at least one of the following regions: the N-terminal amino acids 1-8, the ω-loop amino acids 36-45, the α-helix amino acids 70-74 or the helix turn amino acids 84-89, said method comprising:

(a) transforming a recombinant host cell with an expression vector which contains an enzymatically active subtilisin DNA which upon expression provides for the expression of a subtilisin which does not bind calcium protein;

(b) culturing said host cell under conditions which provide for the expression of the enzymatically active subtilisin mutant; and

(c) recovering the expressed enzymatically active subtilisin mutant from said microbial host.

14. The recombinant method of claim 13, wherein the subtilisin mutant lacks the calcium A binding site.

15. The recombinant method of claim 14, wherein the mutant lacks amino acids 75-83.

16. The recombinant method of claim 15, wherein the mutant has one or more additional mutations in the amino acid sequence.

17. The recombinant method of claim 16, wherein the subtilisin mutant comprises at least one substitution mutation of S221C, N218S, M50F, Y217K, P5S, D41A, K43R, K43N, Q271E, Q2K, Q2W, Q2L, A73L, A73Q, Q206C, Q206V, Q206I, Q206W or S3C.

18. The recombinant method of claim 13, wherein the subtilisin mutant is a subtilisin BPN' mutant.

19. The recombinant method of claim 18, wherein the subtilisin BPN' DNA comprises one or more additional mutations which provide for enhanced thermal stability or which provide for restoration of cooperativity of folding of the subtilisin protein.

20. A recombinant DNA which encodes for a subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein

comprises one or more deletion, substitution or addition mutations in at least one of the following regions: the N-terminal amino acids 1-8, the ω-loop amino acids 36-45, the α-helix amino acids 70-74 or the helix turn amino acids 84-89, and which mutated subtilisin protein retains enzymatic activity and stability.

21. The recombinant DNA of claim 20, wherein the subtilisin DNA is a subtilisin BPN′ coding sequence which lacks the codons encoding amino acids 75-83.

22. The recombinant DNA of claim 21, wherein the subtilisin DNA is a subtilisin BPN′ coding sequence which comprises a codon encoding one or more additional mutations in the amino acid sequence.

23. The recombinant DNA of claim 22, wherein the subtilisin DNA is a subtilisin BPN′ coding sequence which comprises a codon encoding one or more additional mutations in the β-ribbon amino acids 202-219.

24. The recombinant DNA of claim 22 which further comprises a codon encoding at least one substitution mutation of S221C, N218S, M50F, Y217K, P5S, D41A, K43R, K43N, Q271E, Q2K, A73L, A73Q, Q206C, Q206V, Q206I, Q206W or S3C.

FIG. Ia

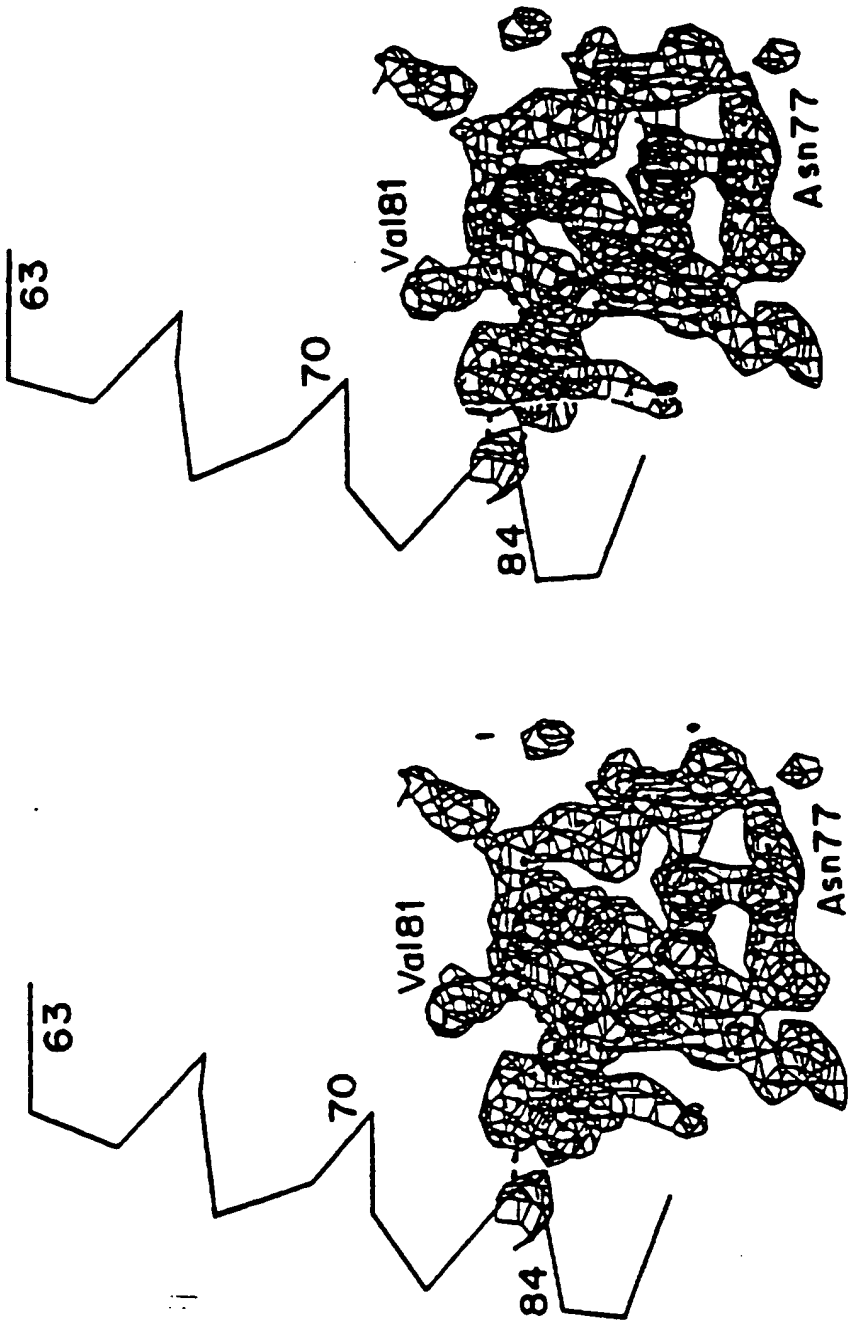


FIG. Ib

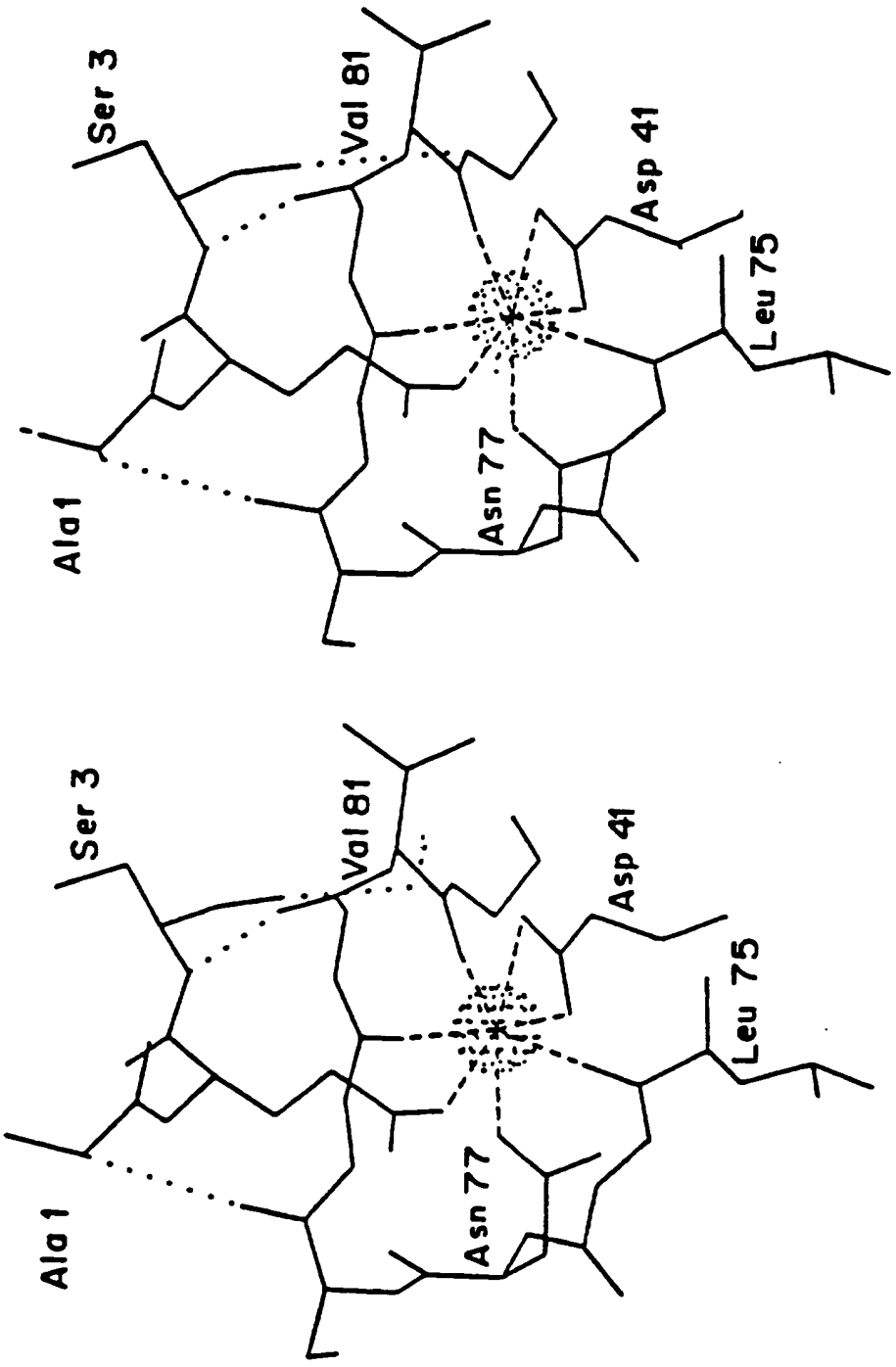

Ser 3
Val 81
Asp 41
Leu 75
Asn 77
Ala 1
Ser 3
Val 81
Asp 41
Leu 75
Asn 77
Ala 1

FIG. 2

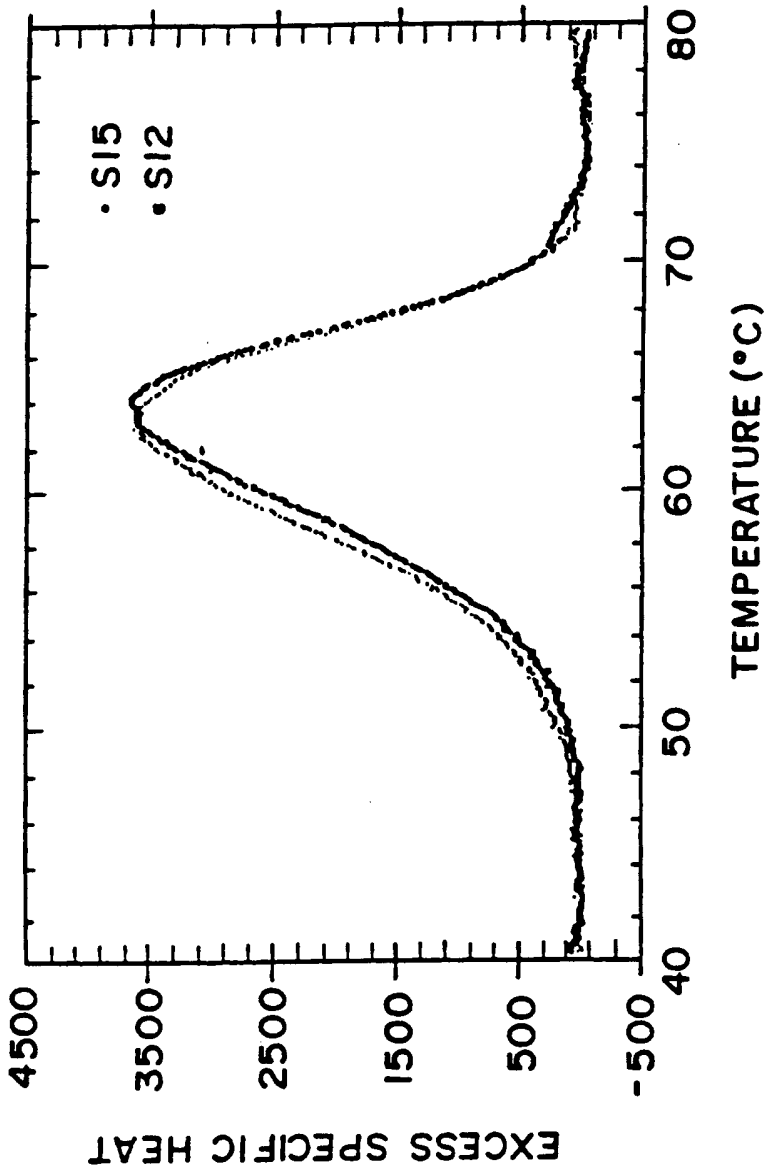

S15
S12
4500
3500
2500
1500
500
-500
EXCESS SPECIFIC HEAT
40
50
60
70
80
TEMPERATURE (°C)

FIG. 3

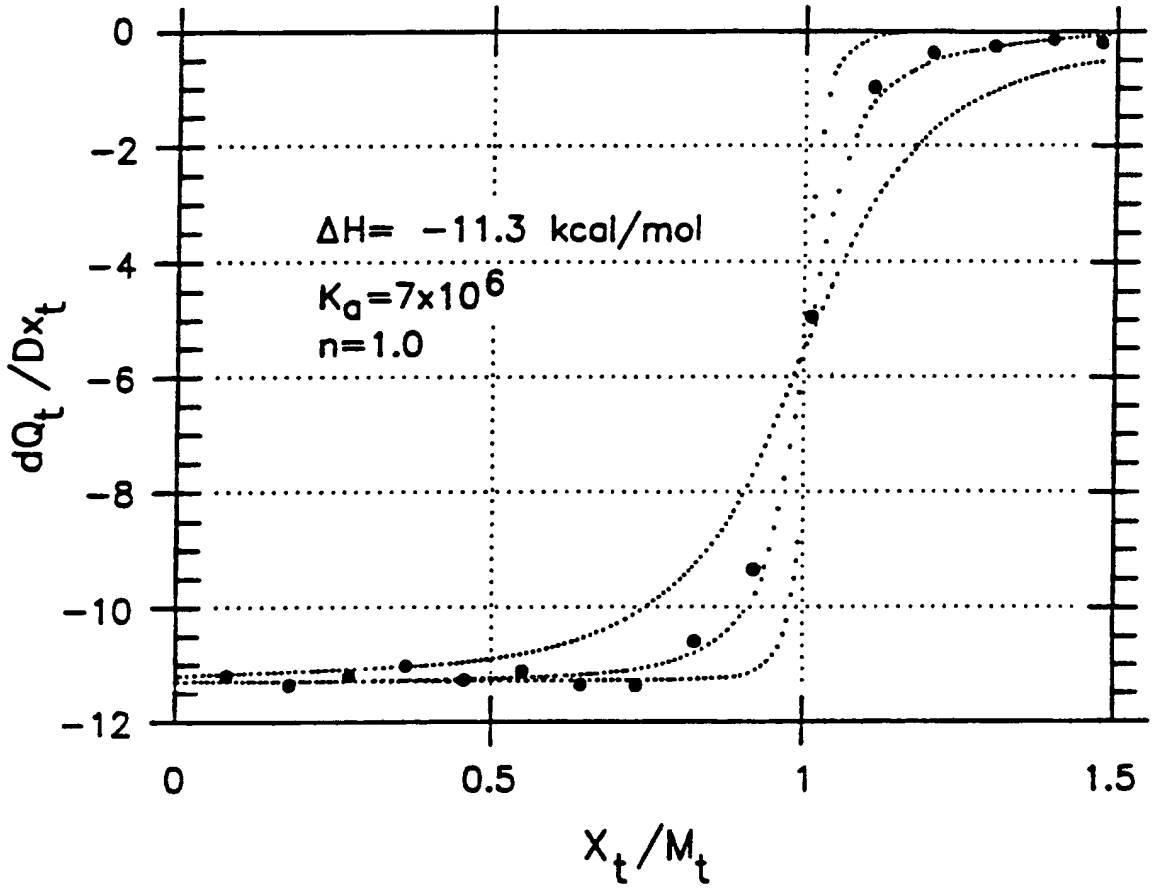


FIG. 4

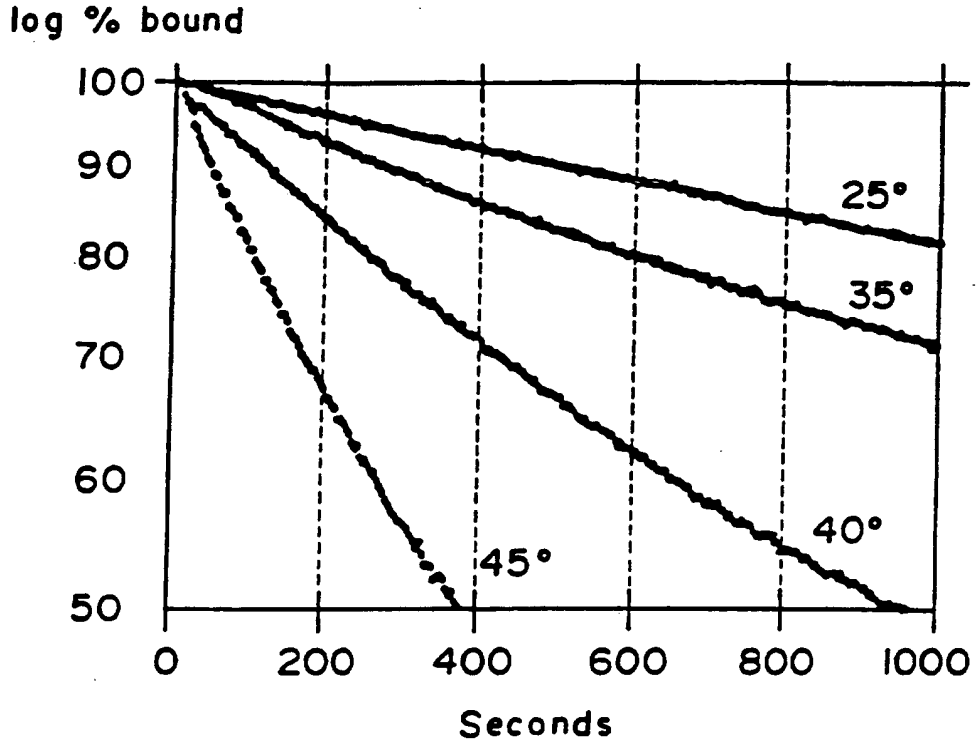

log % bound
100
90
80
70
60
50
0
200
400
600
800
1000
Seconds
25°
35°
40°
45°

FIG. 5a

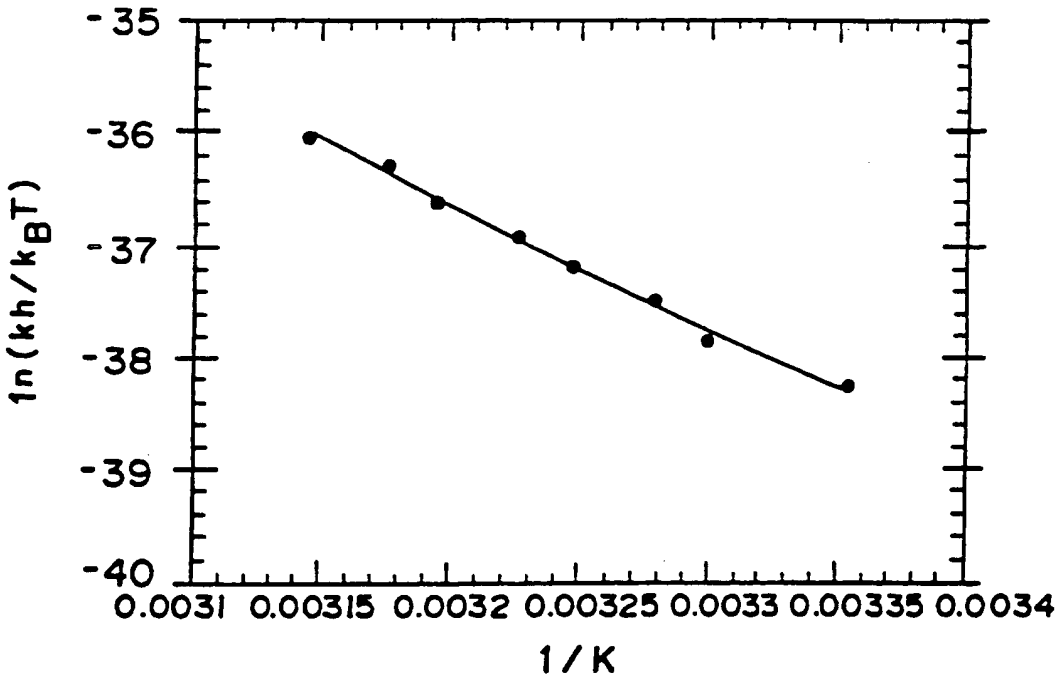

-35
-36
-37
-38
-39
-40
ln(kh/k_BT)
0.0031
0.00315
0.0032
0.00325
0.0033
0.00335
0.0034
1/K

FIG. 5b

FIG. 6a

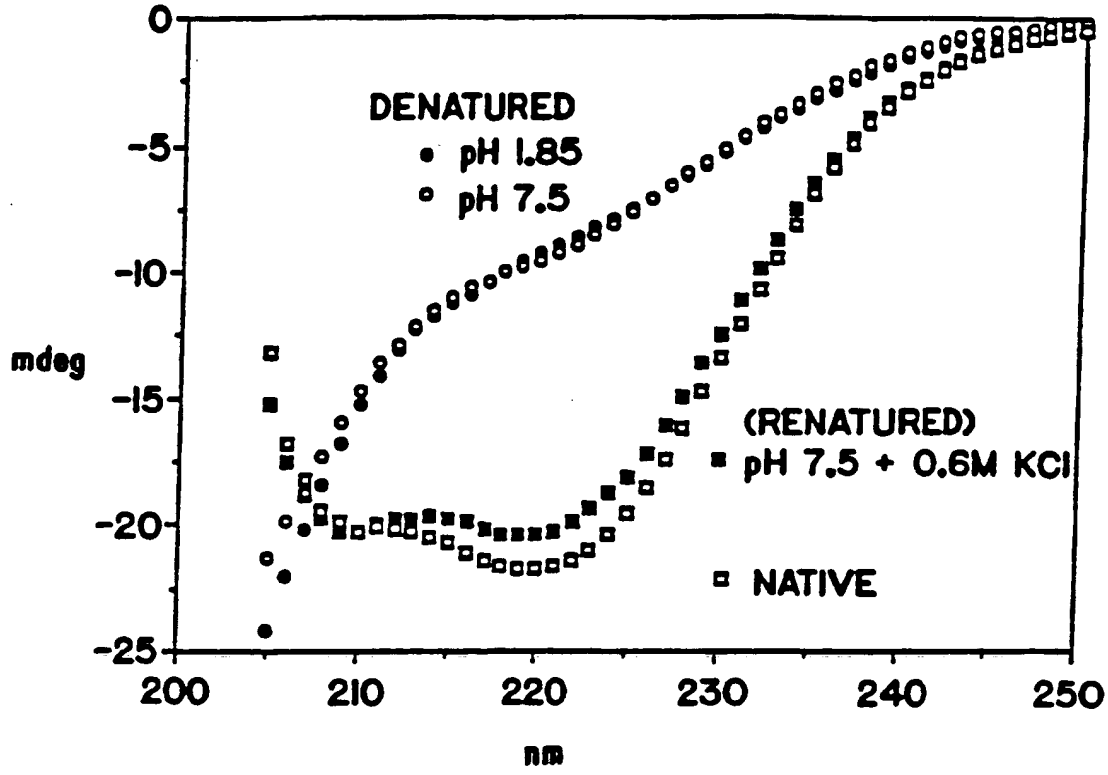

0
-5
-10
-15
-20
-25
mdeg
DENATURED
pH 1.85
pH 7.5
(RENATURED)
pH 7.5 + 0.6M KCl
NATIVE
200
210
220
230
240
250
nm

FIG. 6b

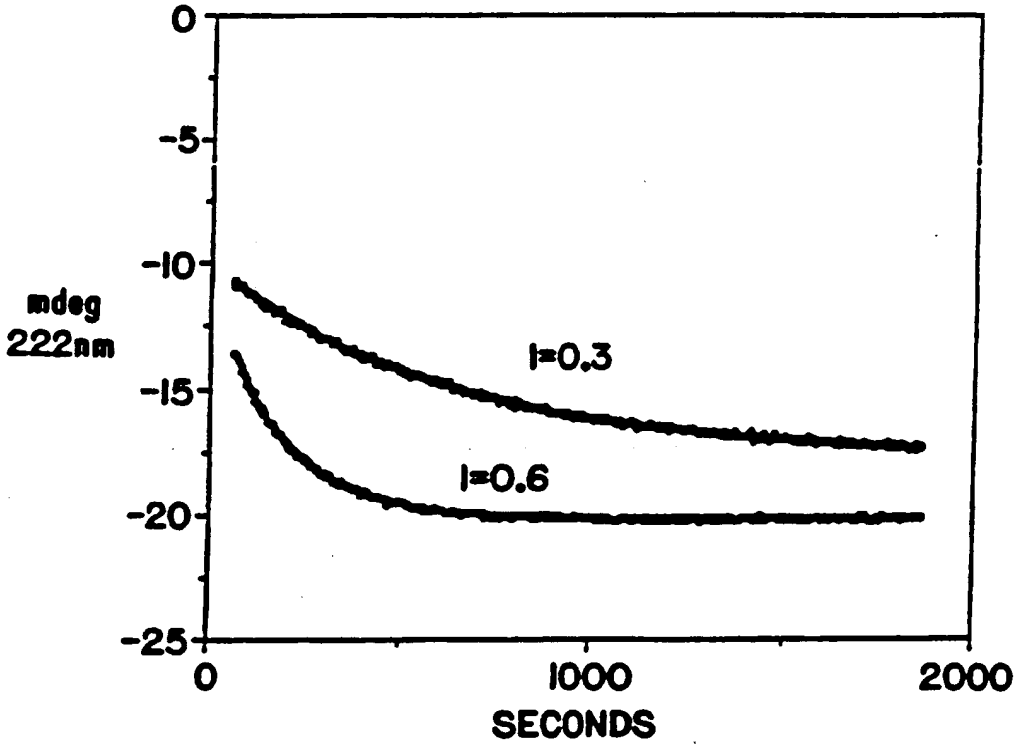

0
-5
-10
-15
-20
-25
mdeg
222nm
I=0.3
I=0.6
0
1000
2000
SECONDS

FIG. 7a

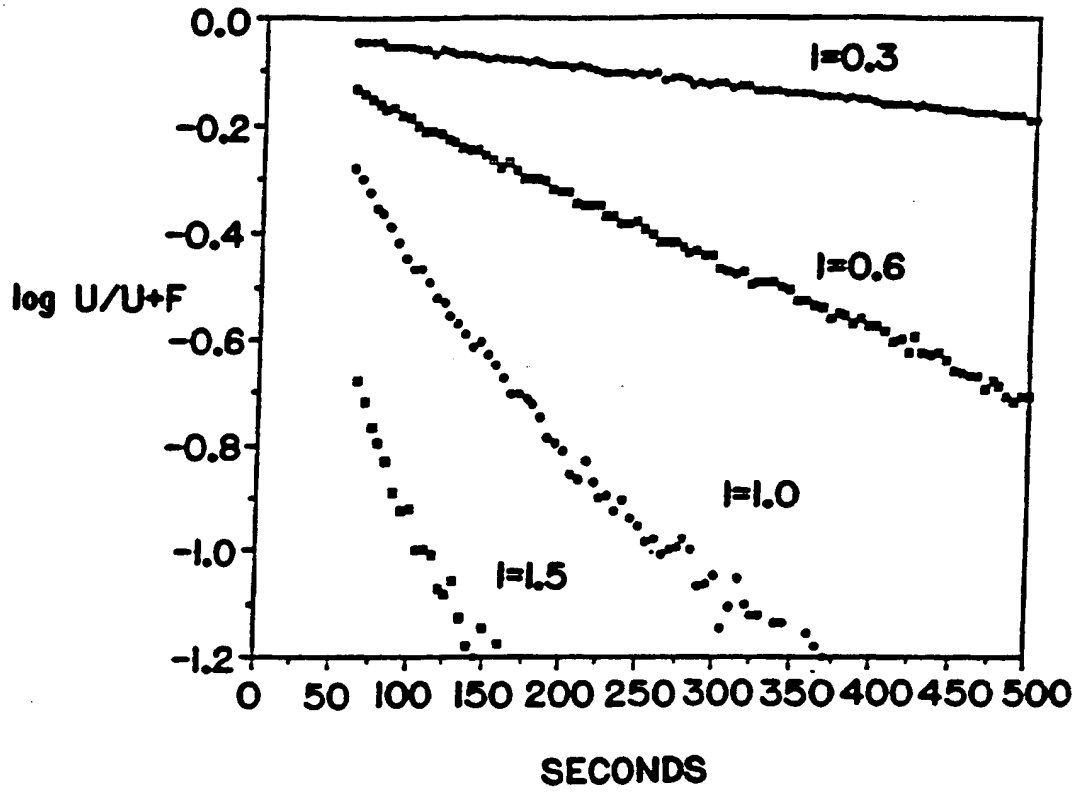

0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
log U/U+F
I=0.3
I=0.6
I=1.0
I=1.5
0 50 100 150 200 250 300 350 400 450 500
SECONDS

FIG. 7b

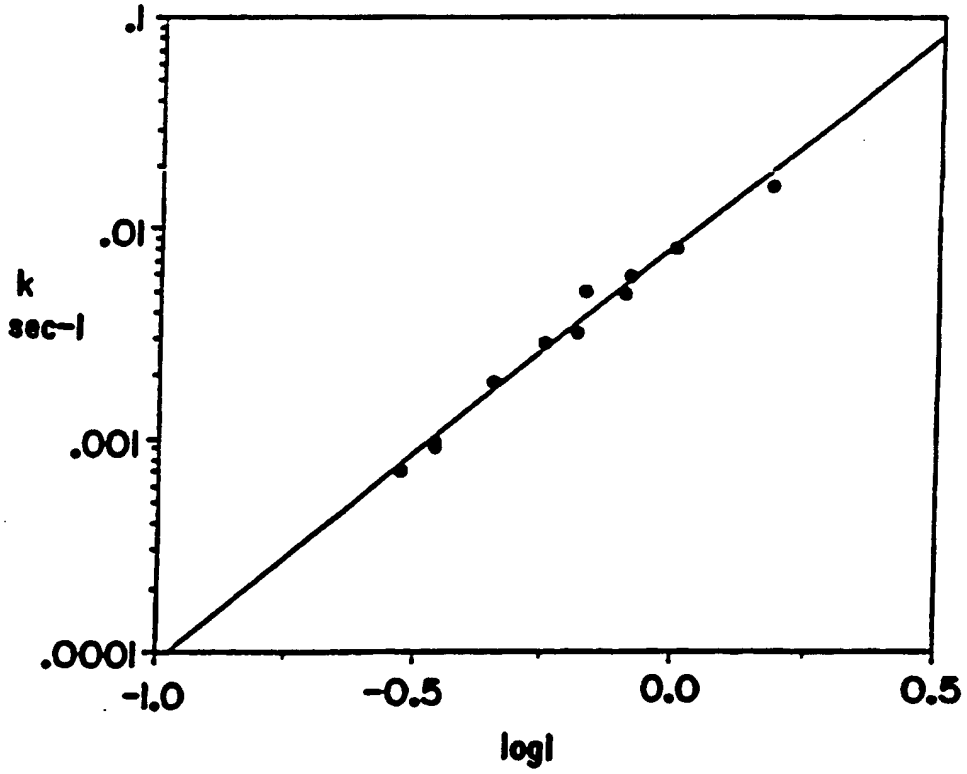
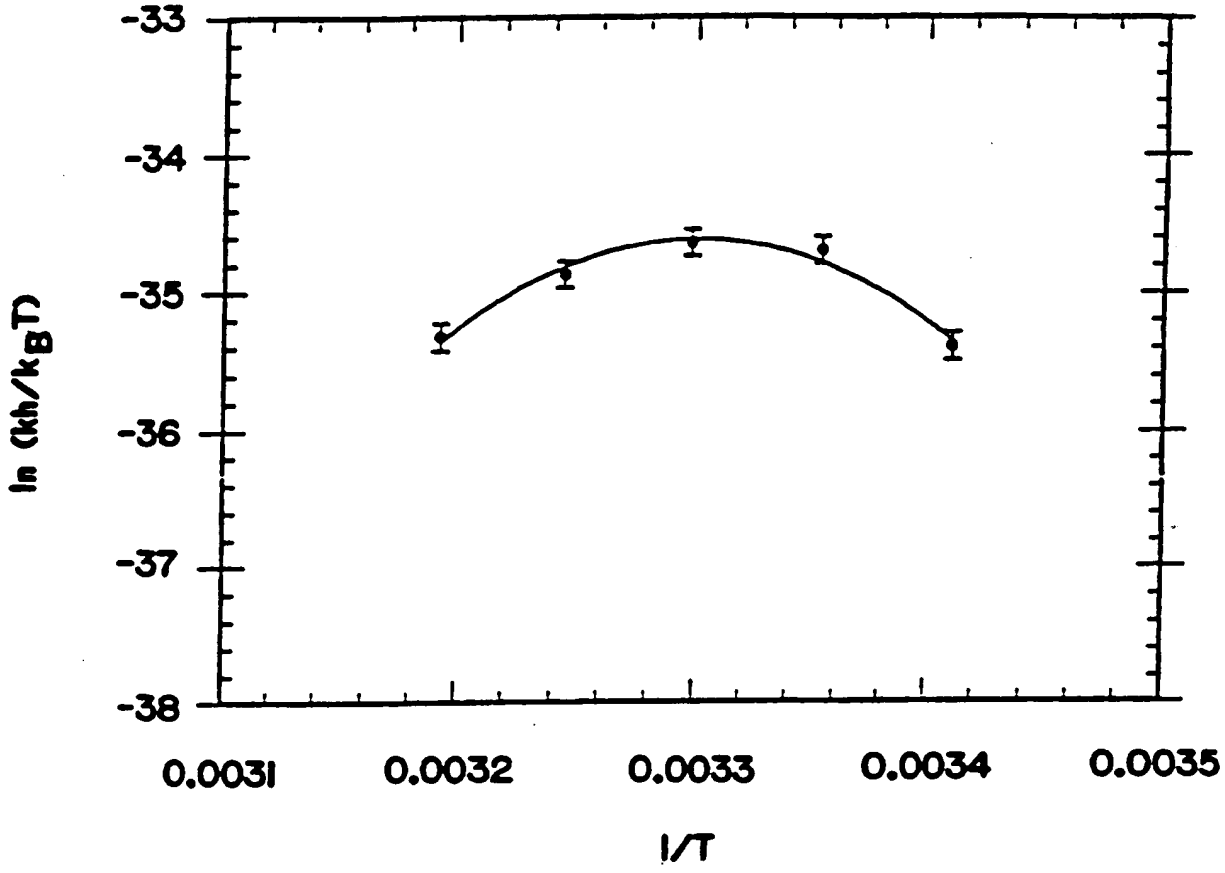

.1
.01
.001
.0001
k
sec-1
-1.0
-0.5
0.0
0.5
logI

FIG. 8



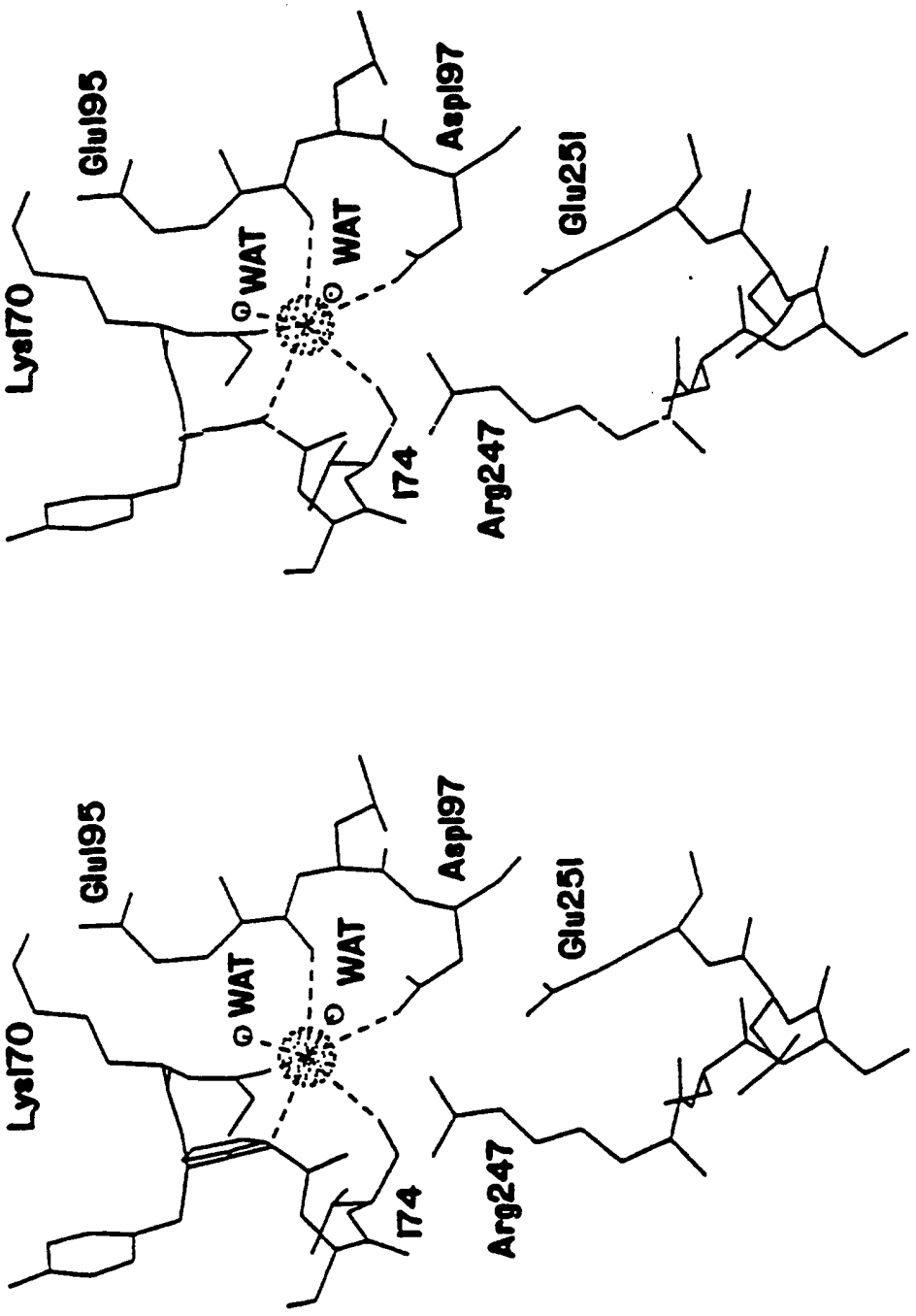

Glu195
Asp197
Glu251
WAT
WAT
Lys170
I74
Arg247
Glu195
Asp197
Glu251
WAT
WAT
Lys170
I74
Arg247

FIG. 9

INTERNATIONAL SEARCH REPORT

Inte mal Application No
PCT/US 95/05520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N9/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEINS STRUCT FUNCT GENET 16 (2). 1993. 205-213. CODEN: PSFGEY ISSN: 0887-3585, GALLAGHER T ET AL 'CALCIUM-INDEPENDENT SUBTILISIN BY DESIGN.' see the whole document ---	1-24
X	BIOCHEMISTRY 32 (39). 1993. 10371-10377. ISSN: 0006-2960, STRAUSBERG S ET AL 'An engineered disulfide cross-link accelerates the refolding rate of calcium-free subtilisin by 850-fold.'	1-3,5, 10-22,24
Y	see the whole document --- -/--	3-9,16, 17,22-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search
20 November 1995

Date of mailing of the international search report
05. 12. 95

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer
Van der Schaal, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY 32 (32). 1993. 8112-8119. CODEN: BICHAW ISSN: 0006-2960, STRAUSBEG S ET AL 'CATALYSIS OF A PROTEIN FOLDING REACTION THERMODYNAMIC AND KINETIC ANALYSIS OF SUBTILISIN BPN INTERACTIONS WITH ITS PROPEPTIDE FRAGMENT.'	1-3, 10-16, 18-22
Y	see the whole document	3-9,16, 17,22-24

Y	BIOCHEMISTRY, vol. 28, 1989 pages 7205-7213, M. PANTOLIANO 'Large increases in general stability for subtilisin BPN' through incremental changes in the free energy of unfolding' see the whole document	3-9,16, 17,22-24

X	WO,A,89 09830 (GENEX CORP) 19 October 1989	1,10-13, 18-20
Y	see claims	5-9,16, 17,22-24

X	EP,A,0 405 901 (UNILEVER PLC ;UNILEVER NV (NL)) 2 January 1991	1,10-13, 18-20
Y	see claims	5,16,17, 22,24

X	WO,A,89 09819 (GENEX CORP) 19 October 1989	1,10-13, 18-20
Y	see claims	3-9,16, 17,22-24

X	WO,A,92 21760 (COGNIS INC) 10 December 1992 see claims; tables 2,3	1,10-13, 18-20

X	WO,A,88 08164 (GENEX CORP) 20 October 1988 see table 2	1,10-13, 18-20

X	PROTEIN ENGINEERING, vol. 4, no. 1, 1990 pages 87-97, C. ERWIN ET AL 'Effects of engineered salt bridges on the stability of subtilisin BPN'' see the whole document	1,10-13, 18-20

X	WO,A,94 02618 (GIST BROCADES NV ;MULLENERS LEONARDUS JOHANNES S (NL); MISSET ONNO) 3 February 1994 see claims	1,10-13, 18-20

	-/--	

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/05520

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 398 539 (AMGEN INC) 22 November 1990 see the whole document	1-3, 13-15, 20,21
A	EP,A,0 405 902 (UNILEVER PLC ;UNILEVER NV (NL)) 2 January 1991 see page 5, line 54	1,13,20
A	WO,A,92 11357 (NOVONORDISK AS) 9 July 1992 see claim 13	1,13,20
A	WO,A,88 08028 (GENEX CORP) 20 October 1988 see the whole document	1,13,20

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/US 95/05520

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8909830	19-10-89	US-A-	5013657	07-05-91
		EP-A-	0409878	30-01-91
		JP-T-	3503602	15-08-91
		US-A-	4990452	05-02-91
		US-A-	5246849	21-09-93
EP-A-0405901	02-01-91	WO-A-	9100334	10-01-91
		JP-T-	4500385	23-01-92
		EP-A-	0405902	02-01-91
		WO-A-	9100335	10-01-91
		JP-T-	4500384	23-01-92
WO-A-8909819	19-10-89	US-A-	4990452	05-02-91
		EP-A-	0409892	30-01-91
		JP-T-	3503604	15-08-91
		US-A-	5116741	26-05-92
WO-A-9221760	10-12-92	US-A-	5340735	23-08-94
		EP-A-	0590012	06-04-94
		JP-T-	6508264	22-09-94
WO-A-8808164	20-10-88	US-A-	4908773	13-03-90
		US-A-	4990452	05-02-91
WO-A-9402618	03-02-94	AU-B-	4700693	14-02-94
		CA-A-	2139928	03-02-94
		FI-A-	950168	10-03-95
		JP-T-	7508887	05-10-95
		PL-A-	307132	02-05-95
EP-A-0398539	22-11-90	AU-B-	618675	02-01-92
		AU-B-	5558090	18-12-90
		CA-A-	2016211	17-11-90
		JP-T-	3505976	26-12-91
		WO-A-	9014420	29-11-90
		US-A-	5397705	14-03-95
EP-A-0405902	02-01-91	WO-A-	9100335	10-01-91
		JP-T-	4500384	23-01-92
		EP-A-	0405901	02-01-91

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]onal Application No
PCT/US 95/05520

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0405902		WO-A-	9100334	10-01-91
		JP-T-	4500385	23-01-92
WO-A-9211357	09-07-92	AU-B-	9121691	22-07-92
		EP-A-	0563169	06-10-93
		JP-T-	6504199	19-05-94
WO-A-8808028	20-10-88	EP-A-	0353250	07-02-90
		JP-T-	2502874	13-09-90
		US-A-	4990452	05-02-91
		US-A-	5260207	09-11-93